CLV. EFFECT OF BACTERIOPHAGE ON OXIDATION-REDUCTION POTENTIALS OF *B. DYSENTERIAE* (SHIGA) CULTURES.

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It has been shown in previous communications that the oxidation-reduction potential curves of peroxide-forming bacteria are entirely different from those of catalase-containing organisms [Hewitt, 1930]. Furthermore, when catalase is added to cultures of peroxide-forming bacteria the oxidation-reduction potential curves obtained are similar in many respects to those of organisms which themselves form catalase [1931, 1]. The behaviour of *B. dysenteriae* (Shiga) is of interest in this connection since it neither forms peroxide nor contains catalase [McLeod, 1930]. It is, therefore, of importance to determine in what respects the oxidation-reduction potentials developed in cultures of *B. dysenteriae* differ from those developed in cultures of peroxide-forming bacteria on the one hand and catalase-containing bacteria on the other.

In addition to these points the opportunity occurs with *B. dysenteriae* to study the effect of the bacteriophage phenomenon on oxidation-reduction potentials. It appeared possible that the destruction and lysis of bacteria by the action of bacteriophage might be accompanied by characteristic changes in the oxidation-reduction potentials of the cultures.

With these several objects in view observations have been made of the potentials developed in *B. dysenteriae* (Shiga) cultures and of the effect of bacteriophage.

Methods.

The methods and apparatus used were those described previously [Hewitt, 1930; 1931, 2]. In each experiment 8 cc. of culture medium were inoculated with 0·1 cc. of a 24-hour broth culture of the rough variant of *B. dysenteriae* (Shiga) and the potentials developed were followed during the active growth of the culture. The author is indebted to Dr C. Todd for a sample of Shiga phage.

Results.

Potentials of cultures without phage.

Plain broth cultures (Fig. 1). In stationary aerobic peptone-infusion broth cultures the potential fell slowly for the first 2 hours (lag period) and more

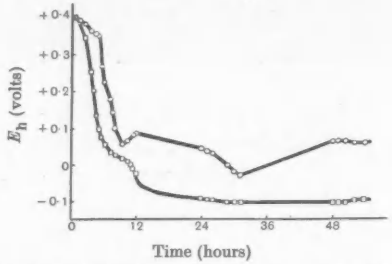

Fig. 1. Infusion broth cultures of *B. dysenteriae* (Shiga).

rapidly for the next 2 hours. The lowest level of potential (− 0·1 v.) was reached after 48 hours' growth and was maintained for several days.

The fall in potential was very sluggish also in cultures which were vigorously aerated by the method previously described [1931, 2]. After a lag period lasting some 4 hours the potential fell to the level + 0·1 v. The fall from this level to + 0·02 v. was very slow as with the stationary aerobic culture. The lowest level of potential (− 0·03 v.) was reached after 30 hours' incubation, and the potential had not risen above the level + 0·06 v. after 54 hours' growth (upper curve, Fig. 1).

B. dysenteriae (Shiga) has, therefore, feeble reducing powers and no evidence was obtained of the high levels of potential corresponding to peroxide formation. The oxidation-reduction potential behaviour resembled that of catalase-containing bacteria except that the reducing powers were less intense.

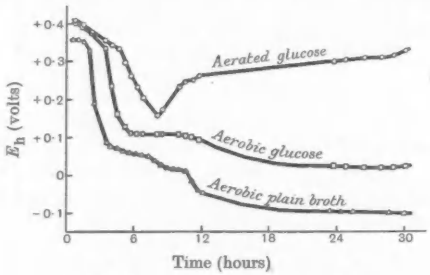

Fig. 2. Cultures of *B. dysenteriae* (Shiga).

1 % glucose broth cultures (Fig. 2). In contradistinction to the behaviour of the other bacteria investigated the potential fell much more slowly in

stationary aerobic glucose broth than in plain broth. The lowest level of potential reached was + 0·02 v. in 25 hours and the potential rose very slowly. With other organisms investigated there has been a rapid fall in potential in glucose broth followed by a rapid rise, then a slight fall and finally a more gradual rise.

In aerated glucose broth cultures the potential reached its lowest level, + 0·16 v., in 8 hours and then rose fairly rapidly. This fall in potential is intermediate between that of haemolytic streptococci (lowest level + 0·2 v.) and that of staphylococci (− 0·01 v.).

Effect of bacteriophage (Figs. 3, 4).

0·1 cc. of Shiga phage was added to an aerobic 7-hour culture of *B. dysenteriae* (Shiga) in infusion broth. After 18 hours' incubation the culture had cleared and was filtered through a small Seitz filter. The bacteriophage was subjected to several passages before it was used.

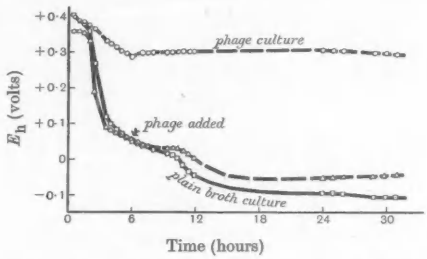

Fig. 3. Stationary aerobic cultures. (Broken lines indicate cultures containing bacteriophage.)

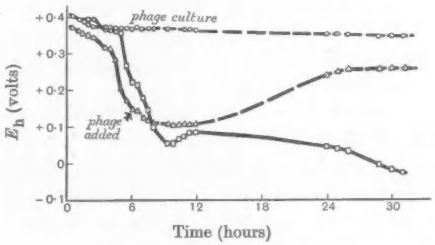

Fig. 4. Aerated broth cultures.

When the phage (0·1 cc.) was added to a culture immediately after inoculation with *B. dysenteriae* the potential fell very little even after 54 hours' incubation, and no visible growth occurred. In neither the stationary aerobic (Fig. 3) nor the aerated cultures did the potential fall below + 0·29 to 0·33 v. It is evident that the dysentery bacilli had not proliferated to any appreciable

extent and that the bacteriophage itself had no detectable effect on the potentials. The bacteriophage had merely inhibited the usual fall in potential accompanying growth of the organisms. In further experiments 0·1 cc. of bacteriophage was added to the culture after 6 hours' incubation. In these experiments the fall in potential slackened after the bacteriophage had been added. After a further 6 hours' incubation in aerated cultures (Fig. 4) and after 18 hours in stationary aerobic cultures the potential had commenced to rise. The bacteriophage appeared to have no effect other than that of inhibiting the proliferation and hence the metabolic changes and fall in potential of the cultures.

Discussion.

B. dysenteriae (Shiga) possesses only feeble reducing powers compared with other bacteria investigated. The potentials of cultures of this organism fall fairly slowly and do not reach the low levels attained by haemolytic streptococci, *C. diphtheriae*, staphylococci and pneumococci [Hewitt, 1930]. Although the Shiga bacillus does not contain catalase, it does not display the electrode potential behaviour of peroxide-forming organisms, but behaves similarly to *C. diphtheriae* and staphylococci, which contain catalase. This general similarity to the catalase-containing organisms is shown in Fig. 5.

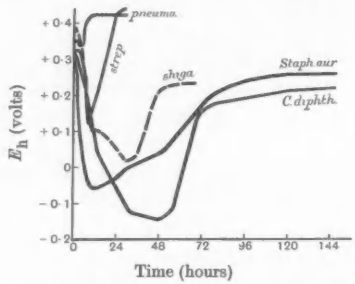

Fig. 5. Aerated broth cultures of different bacteria.

Hydrogen peroxide is formed in many biological oxidation-reduction reactions and cells of many kinds contain catalase and, hence, are able to destroy the toxic peroxide as it is formed. *C. diphtheriae* and staphylococci are examples of this type. Haemolytic streptococci and pneumococci, on the other hand, do not contain catalase and hence peroxide accumulates in aerated cultures of these bacteria. Addition of catalase to their cultures inhibits peroxide formation and certain of the characteristic features of their electrode potential behaviour disappear [Hewitt, 1931, 1]. It is remarkable therefore that the Shiga bacillus, which does not contain catalase, should not behave like a peroxide-forming organism. It is possible that the explanation of the anomaly lies in the fact that *B. dysenteriae* (Shiga) displays peroxidase activity, possibly associated with its cytochrome content [Keilin, 1929] and

thus any peroxide formed in the culture may react, in the presence of peroxidase, with broth constituents, and hence be removed as it is formed.

This is of interest from the point of view of the classification of bacteria. McLeod [1930] has classified bacteria according to their possession or non-possession of catalase and their degree of sensitivity to peroxide. As has been pointed out elsewhere [Hewitt, 1931, 2] other enzymes than catalase must affect the proliferation of bacteria under different conditions of oxygen supply, and it now seems possible that peroxidase activity may be of considerable importance in bacteria which do not contain catalase, as it may provide an alternative method of removing the toxic effects of peroxide accumulation.

Todd [1930] found that in the case of haemolytic streptococci peroxide still accumulated but in smaller amounts when peroxidase was added to the cultures. The question remains unsettled, therefore, as to whether it is the presence of peroxidase or some other difference in respiratory mechanism which accounts for the absence of peroxide formation by the Shiga bacillus.

Bacteriophage itself appears to have no effect on the oxidation-reduction potential of culture media. When added to the cultures at the time of inoculation Shiga phage inhibits the usual fall in potential accompanying the growth and metabolic activities of *B. dysenteriae* (Shiga). Thus the inhibition of growth is reflected in the lack of fall in potential. When the bacteriophage is added after the culture has grown the potential gradually ceases to fall and commences to rise as the bacteria and their metabolic activities are destroyed.

Summary.

1. *B. dysenteriae* (Shiga) possesses relatively feeble reducing powers.

2. Although it does not contain catalase its behaviour resembles that of catalase-containing organisms and not that of the peroxide-forming bacteria.

3. This behaviour may be due to peroxidase activity which is probably of some importance in the classification of bacteria.

4. Bacteriophage itself appears to have no effect on the oxidation-reduction potential but it inhibits the usual fall in potential of *B. dysenteriae* cultures by inhibiting the growth and metabolic activities of the bacteria.

The author's thanks are due to Dr C. Todd, Dr R. G. White and Dr E. W. Todd for their help and encouragement.

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CLVI. EFFECT OF LYSOZYME ON THE OXIDATION-REDUCTION POTENTIALS OF *M. LYSODEIKTICUS* CULTURES.

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In a previous communication [Hewitt, 1931, 1] it is shown that the relatively slow lysis produced by adding bacteriophage to *B. dysenteriae* (Shiga) cultures inhibits the usual fall in potential accompanying the proliferation and metabolic activities of the organisms. The object of the experiments now described was to investigate the effects on the oxidation-reduction potential of the rapid lysis of *M. lysodeikticus* by lysozyme [Fleming, 1922].

Methods.

The oxidation-reduction potentials of cultures were measured by the methods previously detailed [Hewitt, 1930; 1931, 2]. For each experiment an inoculation of 0·1 cc. of a 24-hour broth culture of *M. lysodeikticus* was made into 8 cc. of culture medium. The lysozyme used in most experiments was a sterile dilution (one in ten) of egg-white in saline. In a few experiments human tears were used as a source of lysozyme; these were kindly provided by Dr F. Ridley.

Results.

Cultures without lysozyme (Fig. 1). Horse-flesh infusion broth containing 2 % of "difco" proteose peptone was used as culture medium throughout these experiments.

M. lysodeikticus gives a sparse growth in stationary aerobic broth cultures, but the growth is much more rapid and copious in aerated cultures. These effects are reflected in the corresponding oxidation-reduction potential curves (Fig. 1). In stationary aerobic cultures the potential fell slowly and did not reach the low levels which were attained by the other varieties of bacteria investigated, but in cultures which were continuously aerated by the method previously described [Hewitt, 1931, 2] the potential fell more rapidly and to

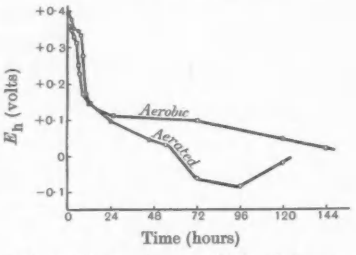

Fig. 1. Broth cultures of *M. lysodeikticus*.

a much lower level. The potentials did not at any time rise to the high levels corresponding to peroxide accumulation. This result is to be expected with an organism displaying such intense catalase activity.

Effect of lysozyme. Sterile egg-white was diluted in 9 volumes of normal saline.

Owing to the sparse growth and slow fall in potential in stationary broth cultures all experiments with lysozyme were conducted with aerated broth cultures.

Fig. 2 illustrates a typical experiment. Five cultures of *M. lysodeikticus*

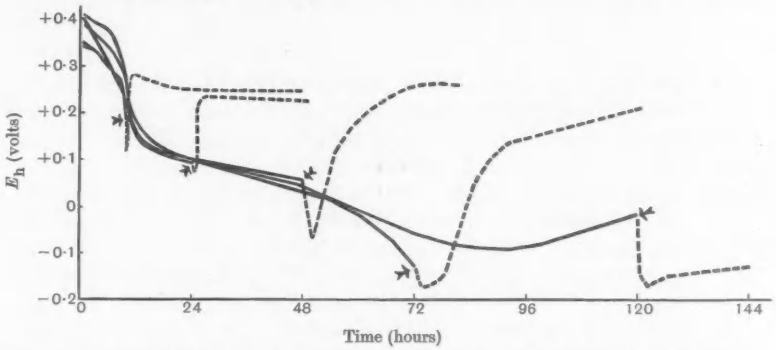

Fig. 2. Aerated broth cultures. Arrows indicate addition of lysozyme (egg-white); broken lines, cultures after addition of lysozyme; continuous lines, cultures before addition of lysozyme.

were aerated vigorously and after 9, 25, 48, 72 and 120 hours' incubation respectively amounts varying from 0·2 to 1·0 cc. of lysozyme (one-tenth egg-white) were added to the different cultures. In each case there was a sudden drop in potential when the lysozyme was added, followed by a rise in potential. The lowest level of potential was reached in each case before the culture had completely cleared and the rise in potential occurred before lysis was complete. The rapidity of the fall and subsequent rise in potential decreased with the age of culture, as can be seen from Fig. 2.

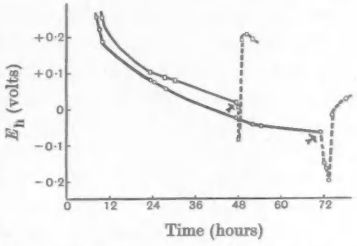

Fig. 3. Aerated cultures. Lysozyme (human tears) was added at the points marked by arrows.

In Fig. 3 are plotted the results obtained with two cultures to which human tears were added after 48 and 72 hours respectively. A fall in potential followed by a rise before lysis was complete was observed as with egg-white, showing that lysozyme from different sources had a similar effect on the electrode potential of cultures.

When lysozyme was added during the first 25 hours' incubation (6 experiments) the average fall in potential was only 0·013 v. and on three occasions the potential during lysis did not fall below that of the original culture. In cultures to which the lysozyme was added after 29 to 120 hours' incubation (10 experiments) the average fall in potential was 0·102 v. The average time required to reach the lowest level of potential after the addition of lysozyme was 4 minutes when the lysozyme was added during the first 25 hours' incubation, and 103 minutes when added subsequently. The lowest level of potential observed in any broth culture of *M. lysodeikticus*, aerobic or aerated, was − 0·139 v. but in a lysed culture a value of − 0·205 v. was attained.

Discussion.

M. lysodeikticus displays marked catalase activity, and no evidence of peroxide formation or of the high levels of potential corresponding to peroxide accumulation was obtained. The general behaviour of the bacteria therefore resembles that of *C. diphtheriae*, *Staphylococcus aureus* and *B. dysenteriae* and not that of the haemolytic streptococcus or the pneumococcus [Hewitt, 1930; 1931, 1, 2]. *M. lysodeikticus* cultures are the only ones so far examined in which the potential falls to a lower level and more rapidly in aerated cultures than in stationary aerobic cultures. Despite the oxidising effect of the increased oxygen supply the bacteria were more easily able to establish reducing conditions when the cultures were aerated, and this was associated with the greatly increased growth under these conditions. Oxygen, therefore, has a negligible effect on the electrode potentials directly, but indirectly its presence enables this obligate aerobe to effect metabolic changes which are impossible when oxygen is absent or present only in small amounts. Evidently *M. lysodeikticus* is deficient in those enzymes which are necessary to effect oxidation-reduction reactions in the absence of air, as shown by the sparse growth and slight fall in potential when the oxygen supply is restricted. Micro-spectroscopic examination revealed that *M. lysodeikticus* like other aerobic organisms contains cytochrome and it seems therefore that this substance, although able to catalyse aerobic oxidations [Keilin, 1929], plays no part in anaerobic reactions, probably because it is fully reduced and inert in the absence of an abundant oxygen supply. Anaerobes are stated not to contain cytochrome.

Table I.

Organism	Lowest levels of potential reached in cultures: Aerobic E_1	Aerated E_2	Difference $E_2 - E_1$
Pneumococcus	− 0·16 v.	+ 0·33 v.	+ 0·49 v.
Haemolytic streptococcus	− 0·17	+ 0·12	+ 0·29
Staphylococcus aureus	− 0·19	− 0·12	+ 0·07
B. dysenteriae (Shiga)	− 0·11	− 0·04	+ 0·07
C. diphtheriae	− 0·21	− 0·15	+ 0·06
M. lysodeikticus	− 0·00	− 0·09	− 0·09

In Table I are summarised the lowest levels of potential reached by different bacteria in ordinary aerobic and in continuously aerated broth cultures.

The results with pneumococcus and haemolytic streptococcus are complicated by the factor of peroxide formation, which limits the fall in potential in aerated cultures. When catalase is present in cultures of pneumococcus the difference $E_2 - E_1$ is only about 0·29 v. and that of the haemolytic streptococcus about 0·23 v. [Hewitt, 1931]. With *S. aureus* growth is more luxuriant in aerated than in stationary aerobic cultures, but the difference is much greater in the case of *M. lysodeikticus*. The oxidation-reduction potential in aerated cultures depends upon a balance between the reductions effected by the bacteria and the direct oxidation of the reducing substances by oxygen. Oxygen appears to have little effect on the potential directly but, on the one hand enables the cell to effect the reductions associated with metabolic changes, and on the other hand oxidises certain of the metabolic reduction products.

It has been shown in a previous communication [Hewitt, 1931, 1] that the slow lysis produced by the addition of bacteriophage to *B. dysenteriae* (Shiga) cultures has the effect of inhibiting the fall in potential associated with the metabolic activities of the organisms, but no other effect was observed. A further effect has, however, been observed during the rapid lysis of *M. lysodeikticus* by lysozyme in aerated cultures. Immediately lysozyme is added to a culture there is a rapid fall in potential. In sixteen experiments the fall ranged from 0 to 0·149 v. (mean 0·068 v.). The fall during the lysis of cultures less than 25 hours old is very rapid but comparatively slight, whereas in older cultures containing more bacteria the fall although less rapid is much greater. The potentials of the cultures cease to fall and commence to rise before lysis has approached completion and the rise is much more rapid in young than in old cultures. Both egg-white and human tears produced these effects. The rise in potential after lysis may reasonably be attributed to the destruction of the metabolic activities of the cell as in the case of bacteriophage, but the explanation of the fall in potential during lysis is more conjectural.

In this connection it is necessary to define current views of the mechanism of establishment of reducing conditions in bacterial cultures. Coulter and Isaacs [1929], in the case of *B. typhosus*, state that "the rôle of the bacteria is the establishment of the anaerobic state" and that "this point of view is opposed to that which regards the potentials of bacterial cultures as a manifestation of reductive substances liberated by the bacteria." It seems more reasonable, however, to assume that neither of these hypotheses is correct, but that the bacteria effect reduction as a natural result of metabolism at the cell surface. To obtain the energy necessary for proliferation, food materials in the broth must be oxidised, and for this purpose free oxygen, combined oxygen, hydrogen acceptors (or electron acceptors) and every type of possible oxidising agent may be utilised by aerobic organisms. The actual agents used by the bacteria depend on the catalysts and enzymes available, and one after

another of the oxidising systems will be reduced as the cell calls its activities into play [Hewitt, 1931, 2]. The view that bacteria can effect reductions only by (i) removal of free oxygen dissolved in the medium, and (ii) liberation of reducing substances from lysed cells, appears to have little biological significance.

Accepting the view of Coulter and Isaacs [1929] bacteria would be regarded as reservoirs of highly reduced material, and the fall in potential during the lysis of the *M. lysodeikticus* would be ascribed to the sudden liberation of highly reducing substances from the lysed bacteria.

An alternative explanation lies in the hypothesis of a temporary increase in the enzymic activity of the bacteria during the lytic process. Penrose and Quastel [1930] have shown that after lysis of *M. lysodeikticus* certain enzymic activities, such as that of catalase, are augmented, whilst others, including those of the dehydrogenases, are almost wholly destroyed. Catalase is presumably contained in the cells and when these are lysed the enzyme is liberated and comes into intimate contact with its substrate. This is in accordance with the observation [Hewitt, 1931, 1] that a freshly washed suspension of *M. lysodeikticus* reacts sluggishly with hydrogen peroxide, whereas a suspension which has been allowed to stand for some time, and probably contains some broken-down cells, gives a brisk effervescence with peroxide.

Since the dehydrogenase activity disappears when the bacteria are lysed it is assumed that this enzymic activity is dependent on the integrity of the cell structure. These results refer to totally lysed cultures, but it is possible that in cultures undergoing the process of lysis there is an intermediate condition in which there is increased enzymic activity displayed by partially lysed bacteria.

The enzymic activities might be represented by the following scheme:

	Catalase activity	Dehydrogenase activity
Before lysis	+	+ +
During lysis	+ +	+ + + +
After lysis	+ + +	±

The increased dehydrogenase activity would occur when the cells were partly broken down so that there would be intimate contact between the enzymes and substrates, but before the cell structure, upon which certain enzymic activities may be assumed to depend, is completely destroyed. It should be remembered in this connection that the fall in potential noted during lysis ceases before lysis is complete. In each experiment the potential had commenced to rise before the culture had totally cleared. This observation affords some support for the hypothesis that the partially lysed cells are capable of a temporary increase in dehydrogenase activity. This temporarily increased activity of bacterial enzymes would be accompanied by intense reducing effects and hence would be reflected in a fall in the potential.

SUMMARY.

1. *M. lysodeikticus* cultures contain catalase and afford no evidence of peroxide formation.

2. Growth is more luxuriant and the fall in potential is greater in aerated cultures than in stationary aerobic cultures.

3. *M. lysodeikticus* contains cytochrome which appears not to catalyse anaerobic oxidation-reduction reactions.

4. On addition of lysozyme (egg-white or human tears) to cultures there is a rapid fall in potential followed by a rise before lysis is complete.

5. The older the culture the slower is the fall and subsequent rise in potential when lysozyme is added.

6. The fall in potential produced by lysozyme is tentatively ascribed to an increase in the enzymic activity (*e.g.* dehydrogenase) of the partially lysed bacteria during lysis.

The author is indebted to Dr R. G. White, Dr E. W. Todd and Dr F. Ridley for their help and interest.

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CLVII. ON AN ENZYME FROM BLOW-FLY LARVAE (*LUCILIA SERICATA*) WHICH DIGESTS COLLAGEN IN ALKALINE SOLUTION.

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It is well known that sclero-proteins are not readily digested by the enzymes of vertebrates; both pepsin and trypsin act upon elastin, collagen is very resistant to trypsin but is digested by pepsin, and keratin is not attacked by either enzyme. However, Stankovic, Arnovlzevic and Matavulj [1929] have described a keratinase from the crops of certain birds of prey and Ssadikov [1927] has claimed that a collagenase is present in ox-pancreas and in some commercial samples of pancreatin. According to earlier workers, the pancreatic enzymes are unable to digest collagen [Sahli, 1924].

Lucilia larvae normally develop in meat or carrion, but they are also a serious pest to living sheep, feeding first in the wool and later boring into the tissues. Under suitable conditions the larvae can consume meat entirely, leaving no trace of connective tissue, and, since they cannot ingest solid particles of meat, the digestion of connective tissue must occur outside the body. The larvae secrete a protease which acts in alkaline solution and persists in the excreta [Hobson, 1931]. An investigation, therefore, was made of the action of the excreta on connective tissue proteins. Their action on keratin was also studied, as a keratinase, if present, would explain how the larvae feed in the wool of sheep and penetrate the skin.

Experimental.

The excreta were obtained by placing washed larvae in a filter and allowing water to drip slowly through the filter for several hours. The product is a dark brown alkaline liquid which consists of diluted excreta probably admixed with saliva; its reaction varies from p_H 8·2 to 8·8. Collagen was prepared from the *tendo Achillis* of an ox and from catgut, elastin from the *ligamentum nuchae* of an ox, and keratin from pure wool. In each case the soluble proteins were removed by washing with water and a brief digestion with trypsin.

The action of the excreta on sclero-proteins.

Preliminary qualitative experiments showed that the excreta dissolved small pieces of collagen and elastin completely, the solution showing an increased formaldehyde titration; no digestion, however, occurred with keratin. The relative activity of the excreta on the different proteins and the results obtained with a pancreatin solution are shown in Table I. The digestions

Table I. *The action on sclero-proteins of the excreta from* Lucilia *larvae and trypsin.*

	Excreta preparation	Pancreatin solution	
Collagen*	12	0	mg. dissolved
Elastin	16	25	
Keratin	0	0	
Gelatin	1·5	1·4	Increase in formaldehyde titration in cc.

* *Tendo Achillis.*

were carried out for 48 hours at 37° at p_H 8·0 in the presence of thymol. Proteolytic activity was determined by formaldehyde titrations of gelatin digestions and the action on sclero-proteins by weighing the residues after digestion and estimating the amounts dissolved. The results show that the excreta dissolve collagen, whereas a pancreatin solution of about the same proteolytic strength has no effect. Elastin, however, is digested more readily by pancreatic trypsin than by the protease in the excreta and neither enzyme attacks keratin. Controls, with enzyme solutions which had been heated for 5 minutes at 100°, showed no action.

Attempted separation of collagenase and protease.

Ssadikov [1927] claimed that the collagenase which he found in pancreatic extracts could be separated from the enzyme which digests fibrin (fibrinase) by adsorption with kaolin or charcoal. Kaolin adsorbed the collagenase leaving the fibrinase in solution, while with charcoal the reverse was found. However, with pancreatin solutions Ssadikov obtained different results; after treatment with kaolin the fibrinase was present in both adsorbate and filtrate, the collagenase disappearing, while with charcoal the fibrinase and collagenase were present in both fractions.

These experiments were repeated with the excreta from *Lucilia* larvae. 8 cc. portions of the excreta preparation were shaken at intervals for 30 minutes with 1 g. of the adsorbent, filtered and aliquots of the filtrate tested upon gelatin and catgut, digestion being measured by formaldehyde titration and loss of weight respectively. In addition, the stability of the enzymes in the excreta was studied by measuring the activity of samples which had been kept for different periods at 37°. The results obtained with two preparations of excreta are given in Table II. It is evident that the treatment with charcoal and with kaolin has reduced the activity towards both gelatin and collagen

Table II. *The stability and adsorption of the enzymes in the excreta of* Lucilia *larvae.*

Exp.	Enzyme and treatment	Time of digestion in hours	Action on gelatin (increase in formaldehyde titration in cc.)	Action on collagen (mg. catgut dissolved)
Excreta preparation A:				
1	Unchanged	18	0·95	20
2	Kept 24 hours at 37°	18	0·9	12·5
3	Treated with kaolin	24	0·55	8
4	„ charcoal	24	0·5	4·5
Excreta preparation B:				
5	Unchanged	18	1·45	22
6	Kept 48 hours at 37°	18	1·05	11·5
7	„ 96 „	18	0·80	5·0
8	Treated with kaolin	18	0·95	9·5
9	„ charcoal	18	0·95	5·0

and, although no absolute separation has been effected, comparison of the results shows that differential adsorption has occurred. In Exps. 1, 8 and 9 the digestion of the gelatin has proceeded to the same extent but the amounts of catgut passing into solution are very different (20, 9·5 and 5·0 mg.). The digestion of collagen by the excreta seems therefore to be due to a separate enzyme which will be referred to as collagenase. Both charcoal and kaolin adsorb the collagenase more completely than the protease, charcoal being the more selective. The collagenase also appears to be less stable than the protease in alkaline solution at 37°. The results obtained by adsorbing the excreta of *Lucilia* larvae with kaolin and charcoal are in fair agreement with those of Ssadikov [1927] for pancreatin samples containing collagenase. However, Ssadikov found that kaolin destroyed the collagenase in pancreatin and obtained different results with glycerol extracts of the pancreas.

The origin of the collagenase.

The present author [1931] has already shown that the protease found in the excreta of *Lucilia* larvae is secreted by the mid-gut, but it is possible that the collagenase may be secreted elsewhere or produced by intestinal bacteria. To investigate this question, larvae were starved until the gut was empty, and the salivary glands, mid-guts and hind-guts from 15 larvae were dissected out, each lot being ground up to an emulsion with one drop of glycerol, diluted with 1 cc. of buffer at p_H 8·4 and tested with a small piece of catgut as substrate. Only the mid-gut preparation digested the collagen, and this showed very weak activity compared with the excreta. Furthermore, a preparation of the gut-contents of feeding larvae was found to digest gelatin actively, but not collagen. These results suggest that the collagenase may be produced by bacteria in the hind-gut, but this possibility was disproved by showing the presence of the enzyme in sterile larvae.

The technique of rearing *Lucilia* larvae aseptically will be described in

detail in a later publication; briefly the method used was to sterilise the eggs with 0·1 % mercuric chloride and rear the larvae on autoclaved brain. The residue from the growth of a batch of larvae shown to be sterile was tested by mixing it with an equal volume of buffer at p_H 8·4, adding thymol and catgut and incubating for 2 days at 37°. On examination the catgut showed definite evidence of digestion, being almost completely dissolved.

The collagenase therefore is produced by the larvae and not by bacteria, and there can be little doubt that it is secreted in the mid-gut. The inactivity of the gut-contents from fed larvae is probably due to adsorption of the enzyme by the food, as adding undigested food from the crop has been found to decrease the action of the excreta on collagen. The high ratio of collagenase to protease found in the excreta may be due to the disappearance of the protease, either by its being used up in digestion of the meal or by being partly readsorbed.

The influence of p_H *on the digestion of collagen by the excreta of* Lucilia *larvae.*

To investigate the effect of reaction on collagen digestion, equal lengths of uniform catgut were digested for 48 hours with the same volumes of an excreta preparation at different p_H values. Aliquots of the excreta were titrated with strong acid and alkali to the required reactions by means of a capillary pipette and mixed with equal volumes of buffer of the same p_H. Digestion was measured by estimating the loss of weight and p_H determined colorimetrically at the beginning and end of the experiment, the mean values being taken. The results are plotted in Fig. 1 and show that the optimum reaction for the

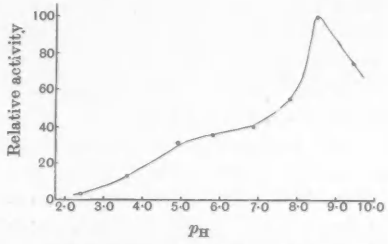

Fig. 1. p_H-activity curve of the digestion of collagen (catgut) by the excreta of *Lucilia* larvae.

collagenase lies between p_H 8·0 and 9·0. This reaction is more alkaline than the optimum found for the digestion of gelatin by the excreta, which was about p_H 7·5 [Hobson, 1931]. The collagenase is well adapted for working in blown meat, in which large amounts of ammonia develop, and the reaction generally lies between p_H 8·0 and 8·5.

Discussion.

The presence of a collagenase in the excreta may play an important part in the growth of *Lucilia* larvae on meat, by digesting the fine strands of connective tissue which surround each muscle fibre and represent to a large extent the structural elements of muscle tissue. The absence of a keratinase is hardly surprising, as this enzyme would only be advantageous in the case of myiasis on sheep, which is exceptional and possibly only a recently acquired habit. When developing in the wool of sheep, *Lucilia* larvae probably feed on the impurities present; if any digestion of keratin occurs, it must be the result of bacterial action.

An enzyme able to digest collagen has been described also by Shinoda [1928] from the gastric juice of the crayfish (*Astacus*). The enzymes from *Astacus* and from *Lucilia* larvae differ in their sensitivity to the reaction; with the gastric juice from *Astacus*, Shinoda found that digestion of collagen occurred over a wide range from p_H 4·0 to 8·0, the actual optimum at p_H 6·7 not being sharply defined; with the excreta from *Lucilia* larvae, the p_H-activity curve (Fig. 1) shows that digestion is limited to a narrow range on the alkaline side of neutrality, the optimum being well marked at about p_H 8·5. Shinoda measured the activity of the gastric juice of *Astacus* at different times during the secretion rhythm against various substrates, but failed to find evidence for the existence of separate proteolytic enzymes. He suggested that, until invertebrate proteases have been separated by modern methods of isolation, reference should be made to "enzyme actions" and not to "enzyme individuals." Shinoda partly based his argument on the fact that the protease from *Astacus*, by digesting collagen in neutral solution, combines to some extent the properties of pepsin and trypsin. However, this is not the case with the protease from *Lucilia* larvae, for adsorption with charcoal decreases the activity of the excreta towards gelatin and collagen to a different extent (Table II) and collagen digestion is, therefore, due to a separate enzyme. Furthermore, Wigglesworth [1928] has shown that the proteolytic enzymes of the cockroach are closely analogous to the corresponding vertebrate enzymes in many respects and was able to separate the tryptase and peptidase fractions by the method used by Waldschmidt-Leitz and Harteneck [1925] for the pancreatic enzymes. There is therefore good evidence that insect proteases include separate individuals which correspond closely to vertebrate trypsin and erepsin.

The reaction products of the collagenase from *Lucilia* larvae could not be determined, as complete separation of the collagenase and protease was not effected. Treatment with dilute acid in the cold or with water at 70° converts collagen into a hydrated form, hydrocollagen, which is digested by trypsin [Ssadikov, 1927], and this may be the end-product of the collagenase from *Lucilia* larvae. It may be noted that the ability of the excreta to digest collagen is not associated with increased action on other resistant proteins;

thus, elastin is digested more readily by trypsin than by the protease from the excreta (Table I).

SUMMARY.

1. The excreta of *Lucilia* larvae contain proteolytic enzymes which digest collagen and elastin but not keratin.

2. The enzyme which digests collagen is produced by the cells of the mid-gut and not by intestinal bacteria, since it occurs in sterile larvae.

3. The excreta digest collagen in alkaline solution, the optimum reaction being at about p_H 8·5. With increasing acidity the activity of the enzyme decreases and almost disappears at p_H 4·0.

4. The separate existence of a collagenase has been concluded from adsorption and stability experiments. The collagenase is less stable than the enzymes acting upon gelatin and is adsorbed to a greater extent by charcoal and kaolin.

I wish to express my appreciation to Dr V. B. Wigglesworth for suggesting this problem and for his active interest in the work. I am indebted to the Empire Marketing Board, for a grant which has financed this work entirely, and to Dr P. A. Buxton, for his interest in this research and hospitality in extending to me the facilities of this Department.

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CLVIII. THE OCCURRENCE OF VITAMIN D IN LAMPREYS (PETROMYZONTIDAE).

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On the assumption that the storage of vitamin D in the livers of fishes is directly related to bone calcification, it might be expected that a decrease in the amount of vitamin D would be observed in passing from the Teleostei, or bony fishes, to the Elasmobranchii, or cartilaginous fishes, the skeleton of which contains very little calcium. The vitamin D storage should, on the same grounds, be still less in the Cyclostomata, a class of primitive vertebrates, distinct from fishes, in which the skeleton shows even smaller traces of calcification than that of elasmobranchs.

A number of species of Elasmobranchii has been investigated by Poulsson [1929, 1931] and by S. and S. Schmidt-Nielsen [1930], who established the presence of vitamin D in the livers, although in much smaller amount than in those of bony fishes, such as the cod. Poulsson suggested that the diffused ossification found in the vertebrae and the calcified deposits in the scales and teeth provided sufficient explanation of the presence of vitamin D. The vitamin D content of Cyclostomata has not previously been investigated by quantitative methods. Matzko [1929] examined one species, *Caspiomyzon wagneri*, which occurs in the Black Sea, in relation to its food value, and found that rats were protected from rickets by the large dose of 0·15 g. per day of the oil from the roasted flesh.

The principal subject of the present investigation has been the lampern, or river lamprey (*Petromyzon fluviatilis*), which is a fairly readily available species. In addition, a few specimens of the sea lamprey (*P. marinus*) have been examined. The liver oils, as well as those obtained from the ovary and from the eviscerated body of these two species, have been examined for vitamin D. The presence of vitamin A has also been investigated in view of the observations made by Mori [1904]. This author found that pills made of the lightly-cooked flesh of the sea lamprey were beneficial in the treatment of the infantile disease "hikan" (xerosis conjunctivae and keratomalacia) prevalent in Japan, although cod-liver oil was more efficacious. McCollum and Simmonds [1917] were the first to recognise this observation as an instance of the cure of vitamin A deficiency. The materials used by Mori have been referred to in the literature, through mistranslation, as "eel-fat" and "chicken-liver."

Experimental.

Material. The lamperns were caught in the River Severn at Tewkesbury in the months of March and April, 1930. At this season they ascend the river from the sea in order to spawn. Eighty-five specimens were received, mostly alive. Immediately after receipt the intestinal tract, liver, and gonads were removed. All the fish appeared to be sexually mature. The sea lampreys were caught in the same neighbourhood in May and June 1930, during the spawning migration. The season was a late and poor one. Only four specimens were received, all in good condition. We are indebted to Dr H. King of this laboratory for his assistance in obtaining these specimens through a local fisherman.

In order to compare elasmobranch fish with the Cyclostomata, some lesser spotted dogfish (*Scyllium canicula*) were examined. These were supplied by Plymouth Marine Biological Station in April and June, 1930. The females, received in April, were immature and the ovaries were rudimentary, but those received in June contained ova from 1 to 2 cm. in diameter.

Extraction of oil. Oils were obtained from the eviscerated bodies, livers, and ovaries of the lampreys, and from the livers of the dogfish. The same procedure was followed in all cases. The minced tissue was treated with 2 % aqueous potassium hydroxide solution (2 cc. to 1 g. of tissue) for 24 hrs. at room temperature, and then for 8–24 hrs. at 37°. After dilution with water, the solution was extracted with ether. Persistent emulsions were broken by the addition of a little alcohol. The extract was washed with water, dried over sodium sulphate, and the ether distilled off, finally under reduced pressure. The oils were stored in stoppered amber bottles in a refrigerator.

Determination of vitamin D. This was carried out by the method described by Bourdillon *et al.* [1931]. Rats rendered rachitic on Steenbock's rachitogenic diet were given comparable doses of the oils investigated and of a standard preparation of irradiated ergosterol, necessary dilutions being made in olive oil. X-ray photographs of the tibiae were taken at the beginning and end of the 14-day period of dosing and the degree of healing was determined by comparison with a calibrated radiographic scale. The antirachitic potencies are expressed in terms of the unit of the Medical Research Council, based on the standard solution issued by them.

Consideration of the "probable error" involved with the number of rats employed [*cf.* Bourdillon *et al.*, 1931] indicates that individual determinations may be subject to considerable error, but the figures obtained for a particular species, by summing the determinations on separate samples of the organs, are capable of indicating differences from another species with reasonable accuracy.

Determination of vitamin A. (*a*) *Biological measurements.* Owing to the small number of animals at our disposal and the limited quantity of most of the oils, only very rough and qualitative estimations of vitamin A contents

Table I. *Vitamin D determinations.*

Species ...	Lampern			Sea lamprey				Lesser spotted dogfish				
Tissue used	Eviscerated body	Liver	Ova	Eviscerated body	Liver (i)	(ii)	Ova	Liver (i) (April)	(ii) (April)	(iii) (June)	(iv) (June)	(v) (June)
Number (weight in g.) of fish used	♂ 11 (205) ♀ 13 (283)	30 (488) 19 (357)	38 (766)	1 (895) 1 (852)	2 (1505)	2 (1541)	2 (1541)	3 (—)	3 (—)	2 (1780)	4 (3180)	1} 1} (1093)
Proportion of tissue to whole fish (%)	87	1·8	12·6	82	3·0	1·9	13·8			8·4	11	11·4
Yield of oil, weight in g.	9·9	0·31	10·6	53·7	10·7	1·2	26·4	104·4	155·3	68	103	62·2
Yield of oil, % of tissue	8·3	3·8	11	7·7	23·7	4·1	12·4	52	57	46	32	53·5
Antirachitic potency of oil, M.R.C. units/cc.	15	53	23·1	60	44	50	102	3·7	7	8·3	5	9
Antirachitic potency of tissue, M.R.C. units/g.	**1·2**	**2·0**	**2·5**	**4·0**	**10·2**	**2·05**	**9·9**	**1·9**	**4·0**	**3·8**	**1·6**	**4·8**
Number of pairs of rats used in each determination	4	3	7	3	2	3	3	5	3	3	3	3

Table II. *Vitamin A determinations.*

Species ...	Lampern						Sea lamprey				Lesser spotted dogfish				
Tissue used	Eviscerated body	Liver (i)	(ii)	(iii)	Ova	Sperm	Eviscerated body	Liver (i)	(ii)	Ova	Liver (i) (April)	(ii) (April)	(iii) (June)	(iv) (June)	(v) (June)
Number (weight in g.) of fish used	♂ 41 (693) ♀ 32 (640)	17} 19} (741)	30 (488)	19 (357)	38 (766)	41 (693)	2 (1505) 2 (1541)	2 (1505)	2 (1541)	2 (1541)	3 (—)	3 (—)	2 (—)	4 (—)	1} 1} (1093)
Proportion of tissue to whole fish (%)	86	1·9	1·5	2·7	12·6	7·6	80	3·0	1·9	13·8	—	—	8·4	11	11·4
Yield of oil, weight in g.	40·5	0·51	0·28	0·03	10·6	0·05	101·7	10·7	1·2	26·4	104·4	155·3	68	103	66·2
Yield of oil, % of tissue	8·4	3·5	3·9	0·3	11	0·1	6·0	23·7	4·1	12·4	52	57	46	32	53·5
Blue value of oil	0·3	12·3	15	16	6·4	3·4	0	Very low	2·8	0·4	0·11	0·05	0·2	0·15	0·4
Blue value of tissue per g.	0·63	10·8	14·2	1·2	17·6	0·085	0	—	2·8	1·24	1·43	0·71	2·3	1·2	5·4
Vitamin A potency: cod-liver oil = 100	8	—	—	—	6·6	—	—	—	—	—	20	—	—	—	11
Number of rats used	2	—	—	—	2	—	—	—	—	—	2	—	—	—	3

of a few oils were made. Growing rats were kept on a diet deficient in vitamin A until loss in weight took place. The oils to be tested were then administered in daily doses of 100 mm.^{3} and cod-liver oil, for comparison, was given in doses of 20 mm.^{3}, and the resumption of growth observed and measured over a period of 30 days. The relative potencies of the oils in the doses given were assumed to be proportional to the percentage increase in weight over this period, and the results are expressed as percentages of the potency of the cod-liver oil used, which had a blue value of 10·2.

(*b*) *Colorimetric measurements.* These are recorded, although we recognise the fact that a direct and quantitative relationship between the intensity of the antimony trichloride reaction and vitamin A content is not established. The blue values, referred to 40 mg. of oil, were determined with a Rosenheim-Schuster colorimeter by the method recommended by the sub-committee of the Pharmacopoeia Commission [1931], but determinations were sometimes made with undiluted oils.

The data of number and weight of fish, weight of tissues, and yield and potency of the oils obtained are given in detail in Tables I and II.

Discussion of results.

Comparison of the vitamin content of lampreys and other species.

The oils obtained from the body, liver, and ovary of both the lampern and the sea lamprey have been found to contain moderate amounts of vitamin D, and the antimony trichloride reaction, supplemented by a few biological tests, indicates that vitamin A is also generally present. The oil with the highest antirachitic potency was that from the ova of the sea lamprey, which was comparable with cod-liver oil. Only two specimens were examined, however, and the figures cannot be regarded as representative. The lesser spotted dogfish likewise yielded liver oils containing vitamins A and D, but in comparatively lower concentration. Since, however, different yields of oil are obtained from corresponding tissues of different species, it is preferable to base the comparison of species on the vitamin content calculated in terms of potency per g. of tissue; these figures are given in the eighth line of the Tables. The livers of lamperns, sea lampreys, and dogfish then appear to possess antirachitic potencies of roughly the same order. Even when account is taken of the relatively greater ratio of weight of liver to total body-weight in the dogfish, the vitamin D stored in its liver does not appear to be significantly greater than that stored in the livers of the lampreys. Moreover, lampreys contain, in addition, vitamin D in the body, which is rich in oil in comparison with that of the dogfish. The dogfish we examined yielded only 0·06 % of oil from the body, and sufficient was not obtained for determination of vitamin D.

Our results are, however, in agreement with those of previous workers who have shown that the livers of cartilaginous fish contain much less

vitamin D than those of bony fish such as the cod. The detailed and exact comparison of the vitamin content of different species is complicated by the influence of the factors of age, food, and sexual condition, as has been demonstrated in the case of the cod [Hess *et al.*, 1929; Drummond and Hilditch, 1930; Hawk, 1930], and it is our opinion that fuller attention must be paid to the variation due to these factors in each species, before definite conclusions can be drawn as to the variation of vitamin D content with species in relation to calcification, or other possible functions. The results we have obtained are based on a limited amount of material, and the evidence they yield must be used cautiously in considering the various problems involved in the calcium and fat metabolism of marine animals.

Relation of size of liver and oil content to sex. The proportion of weight of the liver to the total body weight in the female lampreys was about twice that in the male. The livers of the females were a translucent red, whilst those of the males were creamy and opaque. This difference in colour is probably due to difference in oil content, for the percentage yield of oil from female livers was about one-twelfth of that from male livers. The same difference in the yield of oil from the livers of the two sexes occurred in the sea lampreys. These constant differences in sexually mature lampreys of the same origin appear to be connected with the fat-storing function of the liver, and could perhaps be attributed to the greater transport of fat to the gonads which occurs in the female. Similar relations of size of liver and oil content to sex are known in the cases of the herring, salmon, and cod.

Distribution of vitamins and sex. Recently, investigations on cod have shown that the concentration of vitamin in the liver oil varies inversely with the amount of oil in the liver [Hess *et al.*, 1928, 1929; Drummond and Hilditch, 1930], from which the conclusion has been drawn that vitamins are not transferred to the gonads proportionately with the fat, but to a lesser degree. It might have been expected that comparison of sexually mature fish of different sexes would have shown some striking differences in the vitamin potencies of the liver oils, if it were further assumed that there had been a great difference in the transport of the fat to the gonads in the two sexes. Analysis of the data we have obtained shows, in fact, no evidence of considerable differences of this kind in the contents of either vitamin A or D.

Significance of the presence of vitamin D in lampreys. It is possible to argue that the presence of vitamin D in lampreys and elasmobranch fish is entirely fortuitous, and that it is absorbed from the foods with the fats and accompanies the latter in their distribution in the body. It seems much more attractive to suppose, however, that the vitamin is of physiological importance. Its function might then be explained by the presence of partly calcified structures. The cartilage of the head, for instance, is very hard, and we have been able to demonstrate qualitatively the presence of calcium in the horny teeth of *P. marinus*.

SUMMARY.

The oils extracted from the eviscerated body, liver and ovary of the lampern (*Petromyzon fluviatilis*) and the sea lamprey (*P. marinus*) have been found to contain vitamin D in moderate amount. Data for comparison with a typical cartilaginous fish have been obtained by examination of the liver oil from the lesser spotted dogfish (*Scyllium canicula*). The results indicate that there is little difference between the antirachitic potencies of the tissues of the species examined, although the antirachitic potencies of the livers are much lower than those of the cod. The bearing of the results on a possible correlation between vitamin D content and extent of calcification and on the transference of vitamin D in the body have been considered. Vitamin A is also found to be present in the oils examined.

We have great pleasure in recording our gratitude to Dr O. Rosenheim, who suggested this work, for his constant interest and advice.

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CLIX. THE OCCURRENCE OF SUGAR ALCOHOLS IN MARINE ALGAE. DULCITOL.

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Stenhouse [1844] by isolating mannitol from a number of brown algae established for the first time the occurrence of a sugar alcohol in marine algae. In recent years the subject has been re-investigated by Kylin [1913, 1915], who attempted to estimate the amount of mannitol in different weeds by isolating the crystallised product and weighing it. In the course of an examination of the two brown seaweeds *Pelvetia canaliculata* and *P. canaliculata forma libera* [Haas and Hill, 1929] some difficulty was experienced in isolating the mannitol in crystalline form from the extracts obtained, a fact which was attributed to the presence of the non-crystallisable anhydride mannitan, which substance can also be classed as a sugar alcohol. The presence of both these substances was however established by the preparation of their respective benzylidene derivatives. Kylin [1915] in addition to examining a number of brown weeds also examined four red weeds for the presence of mannitol but in each case failed to find any of this substance. It therefore seemed to us to be of interest to ascertain whether the red alga *Bostrychia scorpioides* contained mannitol, since this weed grows on the salt marsh at Blakeney Point in close association with *P. canaliculata forma libera* and is thus exposed to conditions which are quite abnormal for a red weed. To this end the oven-dried weed was ground, sieved and extracted with boiling 60 % alcohol; the extract, after removal of the alcohol, was treated with basic lead acetate and filtered, and the filtrate was freed from lead by sulphuric acid. The resulting solution was then evaporated over a water-bath in the presence of calcium carbonate to ensure a neutral reaction. The residue thus obtained was a brown opaque pasty mass; it was extracted with boiling absolute alcohol, and the extract on evaporation gave a clear brown syrup, which, on standing for some weeks, deposited crystals. By the gradual addition of an equal volume of alcohol, in small quantities at a time with constant stirring, the crystals were separated from the brown supernatant fluid; they were found to be readily soluble in water but insoluble in alcohol and were accordingly dissolved in water, decolorised with charcoal and crystallised three times from alcohol and water, when they were obtained as glistening

monoclinic prisms melting at 186–187° (uncorr.). They gave the following result on analysis:

	C %	H %
Found	39·85	7·86
Calc. for $C_6H_{14}O_6$	39·56	7·69

When mixed with an authentic sample of dulcitol the melting-point remained unaltered but when mixed with a sample of mannitol of M.P. 163–165° the resulting mixture melted at 152–156°.

These facts in themselves are sufficient evidence that the substance was dulcitol but, to place the matter beyond doubt a small quantity was oxidised with nitric acid and was found to yield mucic acid M.P. 213°.

The occurrence of dulcitol has not previously been recorded in seaweeds, and this is indeed the first time that any sugar alcohol other than mannitol, or its anhydride, has been observed to occur in these plants. It is proposed to examine a number of other red weeds for this substance, but in the meantime it is worthy of note that the occurrence of dulcitol in a group of seaweeds which are known to contain galactose as a cell-wall constituent forms a striking parallel to the occurrence of mannitol in the brown weeds whose cell-wall has been shown to contain mannuronic acid [Bird and Haas, 1931].

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CLX. A PRELIMINARY NOTE ON THE NITROGEN METABOLISM OF SEAWEEDS. GLUTAMIC ACID PEPTIDE.

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(*Received July 27th, 1931.*)

In the course of an examination of the carbohydrate metabolism of certain brown seaweeds [Haas and Hill, 1929] it was found on testing concentrated extracts for sugars by means of Fehling's solution that the solution acquired a mauve tint. On investigation this was shown to be due to the presence of a substance in the extracts which gave a biuret reaction. The isolation of this substance has now been carried out on a somewhat larger scale than was at that time possible.

1 kg. of dried ground *Pelvetia canaliculata*, previously extracted with light petroleum, was covered with boiling water and kept warm over a small flame for about an hour; the weed was then strained through a fine wire sieve and treated a second time as before. The combined filtrates from the two extractions were then passed through calico and poured into an equal volume of absolute alcohol; the mucilaginous brown precipitate thus produced was strained through silk, and the liquid passing through was precipitated with basic lead acetate. After removing the precipitate the colourless solution was freed from lead by means of sulphuric acid, and the solution, while still acid with acetic acid, was treated with a slight excess of mercuric acetate and left to stand overnight. The precipitate A was then filtered, washed free from acid and decomposed with hydrogen sulphide; after removing the lead sulphide the solution was evaporated to small bulk under reduced pressure and finally dried in a vacuum. Thus prepared the product was a light yellow transparent syrup which gradually hardened to a resinous deliquescent solid (P). This substance was insoluble in organic solvents but was readily soluble in water to give an acid solution which on neutralisation however gave no precipitate with silver nitrate.

As the solid showed no tendency to crystallise after repeated attempts had been made to induce it to do so, it was decided to hydrolyse it, more especially as its general character suggested that it was a polypeptide.

Hydrolysis of the peptide (P).

To this end 1·8 g. of the substance was dissolved in 10 cc. of water and heated with 20 cc. of concentrated hydrochloric acid for 3 hours at 105°; the solution was evaporated to dryness over a water-bath and the residue taken up with water, filtered from some black solid and once more evaporated to dryness, when it weighed a little over 2 g. This material, dissolved in the least volume of concentrated hydrochloric acid, yielded 1·7 g. of crystalline solid which on analysis proved to be glutamic acid hydrochloride.

	C %	H %	N %	Cl %
Found	32·53	5·30	7·28	20·41
Calc. for $C_5H_9O_4N.HCl$	32·69	4·90	7·63	19·89

The mother-liquor from the glutamic acid hydrochloride weighing about 0·6 g. contained ammonium chloride; it was treated with freshly precipitated silver carbonate and, after filtering and saturating with hydrogen sulphide, evaporated to dryness in a vacuum. The resulting syrup on drying yielded a colourless resinous mass which was now free from chloride but still yielded ammonia in quantity on addition of dilute caustic soda in the cold and gave an immediate crystalline precipitate with platinic chloride, showing it to be an ammonium salt. It contained 12·72 % of nitrogen but owing to the limited amount of material available it could not be further identified.

Characterisation of the peptide (P).

That the substance was a peptide was shown by the fact that its amino-nitrogen content was increased eightfold after hydrolysis, from which it may be concluded to be an octapeptide.

Determinations of total and amide nitrogen gave the figures 15·07 % and 3·62 % respectively. In addition it was found that the substance had a strong acid reaction which was not increased by the addition of a neutral solution of formaldehyde. Heating over a boiling water-bath with excess of sodium hydroxide and titrating back the excess of alkali gave an equivalent of 209·2.

These figures coupled with the fact that after hydrolysis there remains, in addition to glutamic acid, a certain amount of material which has not as yet been identified make it unsafe to speculate further upon the probable constitution of the peptide, more especially as it has not been found possible as yet to effect any real purification, and the substance is known to contain a trace of ash. It is however hoped to subject this material to further study when more is available.

Occurrence of the peptide in other algae.

On extending the search for this peptide to other brown seaweeds it was found that most of those examined gave the biuret reaction but that the intensity of the reaction fell off with the depth of immersion of the weed, as

may be seen from the following table, in which the number of + signs indicates approximately the relative strength of the biuret reaction obtained.

Pelvetia canaliculata f. libera	+ +
Pelvetia canaliculata	+ +
Fucus vesiculosus	+
Fucus serratus	+
Ascophyllum nodosum	+
Halydris siliquosa	Weak
Himanthalia lorea	Absent
Laminaria spp.	Absent

Extracts prepared from the following green and red weeds all failed to give the biuret reaction: *Enteromorpha intestinalis* (both fresh water and sea water), *Ulva lactuca*, *Chaetomorpha* sp. (fresh water), *Bostrychia scorpioides*, *Chondrus crispus*, *Polysiphonia fastigiata* and *Rhodymenia palmata*.

Physiological significance of the peptide.

In attempting to account for the physiological significance of this substance it is worthy of note that polypeptides, with the possible single exception of glutathione, are generally regarded as transition products which only occur occasionally as intermediate products under conditions of somewhat abnormal metabolism, whereas the evidence so far obtained indicates that it is a stable substance of normal occurrence.

It may be recalled that asparagine and glutamine, which also give the biuret reaction, have been found in seedlings belonging to a large number of widely diverging families, but in such plants these substances appear to owe their presence to conditions of carbohydrate starvation, due to insufficient illumination or other conditions tending to reduce the supply of carbohydrate. Whether the conditions of intermittent desiccation to which the brown seaweeds are exposed and the consequent interruption in photosynthesis are sufficient to account for the appearance of this substance it is not as yet possible to say, though the diminution of the intensity of the reaction with increasing normal depth of immersion of the weeds appears to support this view. It is intended to investigate this matter.

Occurrence of trimethylamine.

The filtrate from the precipitate A (p. 1472), after being freed from mercury with hydrogen sulphide and from sulphuric acid by means of baryta, was evaporated under reduced pressure. The resulting syrup was rendered alkaline with sodium hydroxide and steam-distilled; the distillate was collected in hydrochloric acid and then evaporated to dryness. The residue after drying in a desiccator was extracted with chloroform, and the extract on evaporation left a small quantity of material which gave a crystalline precipitate with a solution of iodine in potassium iodide in the presence of hydrochloric acid; in addition, it gave a brown crystalline precipitate with gold chloride solution containing sodium bromide, tests which, coupled with the characteristic smell

given off on the addition of sodium hydroxide, establish the presence of trimethylamine.

SUMMARY.

1. A preliminary survey of the seaweeds growing round the coast of Britain has shown that the brown weeds exhibit certain peculiarities in their nitrogen metabolism which distinguish them from the green and red weeds growing above and below them.

2. Aqueous extracts of certain brown seaweeds, after appropriate treatment, give a marked biuret reaction, the intensity of which diminishes with the depth of immersion at which the weeds grow.

3. The material responsible for this reaction has been isolated and proves to be an octapeptide of glutamic acid.

4. No similar product has been found in the red or green weeds.

The authors desire to express their indebtedness to Miss M. Parke for supplying them with material.

REFERENCE.

Haas and Hill (1929). *Biochem. J.* **23**, 1000.

CLXI. THE BIOLOGICAL VALUES OF THE PROTEINS OF BREADS BAKED FROM RYE AND WHEAT FLOURS ALONE OR COMBINED WITH YEAST OR SOYA BEAN FLOUR.

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In the course of a study of the nutritional values of various Polish foodstuffs, carried out by us in 1929 and 1930 on behalf of the Flour and Bread Committee of the Polish Ministry of Interior, the biological values of the proteins ($N \times 6{\cdot}25$) of various breads commonly consumed in Poland and of several experimental ones have been ascertained. It is thought worth while to present here such of the results as may be of more general interest.

The biological analysis of 17 products is discussed in the present paper. These may be divided into three groups, (1) various breads commonly consumed in Poland; (2) experimental breads; (3) soya bean flour and baker's yeast.

Breads commonly used were purchased from the Municipal Bakeries in Warsaw. Experimental breads were baked in the Department of Technology of Fermentation and Foodstuffs, Warsaw Technical Institute, under the supervision of Prof. W. Iwanowski who co-operated with us.

In the preparation of the experimental breads we have been interested in combinations of rye and wheat flours with varying amounts of baker's yeast and of soya bean flour. The two latter substances have recently attracted considerable attention as highly concentrated sources of cheap vegetable protein.

The baker's yeast used as admixture was a brand well known in Poland. The soya bean flour "Edelsoja" was purchased from the Oesterreichische Soja-Aktiengesellschaft in Vienna. It is manufactured under a secret process (Berczeller) and is stated by the manufacturers to keep indefinitely without becoming rancid although it contains about 20 % of fat.

The nutritional value of this product and especially its vitamin and mineral contents have been discussed by one of us in a separate publication [Kon, 1930]. As we have made no direct comparisons we do not know whether the biological value of the proteins of the soya bean flour used in the present study is identical with or different from that of the proteins originally present in the soya bean.

Experimental.

The numerical method proposed by Osborne, Mendel and Ferry [1919] was used for the estimation of the biological value of the proteins of the various products studied. The rats were kept singly in cylindrical cages on screens and

Table I.

No.	Product	Protein content* (N × 6·25) %	Remarks
1	White wheat bread	11·0	Baked by Prof. Iwanowski. 280 g. of white wheat flour, 140 cc. of 2½ % solution of NaCl and 5 g. fresh baker's yeast to the loaf
2	Wheat rolls	11·5	From the Municipal Bakeries
3	White wheat-yeast bread I	14·1	Baked by Prof. Iwanowski. 280 g. of white wheat flour, 140 cc. of a 2½ % solution of NaCl and 90 g. of fresh baker's yeast to the loaf
4	White wheat-yeast bread II	15·8	Baked by Prof. Iwanowski. 280 g. of white wheat flour, 150 cc. of a 2½ % solution of NaCl and 140 g. of fresh baker's yeast to the loaf
5	Soya flour-wheat bread I	14·7	Baked by Prof. Iwanowski. 280 g. of a mixture of white wheat flour (90 %) and soya bean flour† (10 %), 150 cc. of a 2½ % solution of NaCl and 5 g. of fresh baker's yeast to the loaf
6	Soya flour-wheat bread II	18·5	Baked by Prof. Iwanowski. 280 g. of a mixture of white wheat flour (80 %) and soya bean flour† (20 %), 160 cc. of a 2½ % solution of NaCl and 5 g. fresh baker's yeast to the loaf
7	Rye bread from 70 % rye flour	6·6	From the Municipal Bakeries. Prepared by acid fermentation. (No yeast added, fermentation started by the addition of fermented dough from previous baking.)
8	Crust of bread No. 7	6·6	
9	Crumb of bread No. 7	7·0	
10	Rye bread from 70 % rye flour	6·3	Baked by Prof. Iwanowski. Same method as for bread No. 7
11	Rye-yeast bread from 70 % rye flour	8·6	Baked by Prof. Iwanowski. 280 g. 70 % rye flour, 140 cc. of a 3 % solution of NaCl and 50 g. of fresh baker's yeast to the loaf
12	Soya flour-rye bread I	10·6	Baked by Prof. Iwanowski. 280 g. of a mixture of 70 % rye flour (90 %) and soya bean flour† (10 %), 160 cc. of a 3 % solution of NaCl and 5 g. of fresh baker's yeast to the loaf
13	Soya flour-rye bread II	14·8	Baked by Prof. Iwanowski. 280 g. of a mixture of 70 % rye flour (80 %) and soya bean flour† (20 %), 170 cc. of a 3 % solution of NaCl and 5 g. of fresh baker's yeast to the loaf
14	Military bread (rye)	9·1	From the Municipal Bakeries. 82 % rye flour. No yeast. (Same method as for bread No. 7)
15	Country dark rye bread (whole-meal)	9·9	Representative sample of country bread baked from home-milled rye flour (approx. 100 %), prepared by acid fermentation
16	Soya bean flour	45·5	A commercial preparation containing about 20 % fat, marketed under the name of "Edelsoja" by the Soja-Aktiengesellschaft in Vienna
17	Baker's yeast	45·6	Same brand as used for admixture to all the yeast breads described above

* On the dry basis. † As described under 16.

the diets were offered in glass feeders with metal hoods to prevent scattering. All diets containing raw starch were mixed with water and heated on the water-bath until dextrinised. Diets containing only bread and the salt mixture were mixed with water without cooking. Tap-water was offered *ad lib.* in glass bulbs. Each rat was given daily, separately from the diet, 3 drops of cod-liver oil and 0·15–0·2 cc. of an Osborne and Wakeman [1919] watery extract from brewer's yeast equivalent to 0·3–0·4 g. of dry yeast and containing 4·8–6·4 mg. N. The extract was omitted in the case of diets containing an abundance of vitamin B complex.

Potato starch was used in the compounding of the rations as it can be obtained in a state of greater purity (lower nitrogen content) than other commercial starches.

Every day the food residues were removed from the food cups, combined with any scattered food, collected on filter-paper on the trays and dried to constant weight. On Saturdays double rations were issued, no cleaning being done on Sundays. The experiments were, on the average, of 30 days' duration.

In Table I a list of the various products analysed and their nitrogen contents are given. Table II presents the composition of the various diets used in

Table II. *Composition of diets.*

All percentages on dry basis.

No.	Diet		%	%	%	%
1	White wheat bread	Protein (N × 6·25) level	8·6	10·4	—	—
		Bread	76·2	95·0	—	—
		Butter fat	10·0	—	—	—
		Salts*	5·0	5·0	—	—
		Potato starch	8·8	—	—	—
2	Wheat rolls	Protein (N × 6·25) level	6·4	8·2	10·9	—
		Rolls	53·0	70·5	95·0	—
		Butter fat	10·0	10·0	—	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	32·0	14·5	—	—
3	Wheat-yeast bread I	Protein (N × 6·25) level	9·1	10·4	12·9	—
		Bread	61·5	71·0	95·0	—
		Butter fat	10·0	10·0	—	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	23·5	14·0	—	—
4	Wheat-yeast bread II	Protein (N × 6·25) level	8·3	9·6	12·3	—
		Bread	51·2	63·3	76·0	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	33·8	21·7	9·0	—
5	Soya flour-wheat bread I	Protein (N × 6·25) level	8·1	10·2	11·9	—
		Bread	54·4	68·0	81·6	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	30·6	17·0	3·4	—
6	Soya flour-wheat bread II	Protein (N × 6·25) level	8·5	10·1	15·3	—
		Bread	43·2	54·0	81·0	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	41·8	31·0	4·0	—

* Steenbock's salt mixture No. 40.

Table II (*contd.*).

No.	Diet		%	%	%	%
7	70 % rye bread	Protein (N × 6·25) level	6·4	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
8	Crust of bread No. 7	Protein (N × 6·25) level	6·3	—	—	—
		Crust	95·0	—	—	—
		Salts	5·0	—	—	—
9	Crumb of bread No. 7	Protein (N × 6·25) level	6·7	—	—	—
		Crumb	95·0	—	—	—
		Salts*	5·0	—	—	—
10	70 % rye bread	Protein (N × 6·25) level	6·0	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
11	Rye-yeast bread	Protein (N × 6·25) level	8·2	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
12	Soya flour-rye bread I	Protein (N × 6·25) level	8·0	10·2	—	—
		Bread	75·5	95·0	—	—
		Butter fat	10·0	—	—	—
		Salts*	5·0	5·0	—	
		Potato starch	9·5	—	—	—
13	Soya flour-rye bread II	Protein (N × 6·25) level	8·1	10·1	11·9	—
		Bread	54·0	67·6	81·1	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Potato starch	31·0	17·4	3·9	—
14	Military bread	Protein (N × 6·25) level	8·6	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
15	Country whole-meal rye bread	Protein (N × 6·25) level	9·7	—	—	—
		Bread	95·0	—	—	—
		Salts*	5·0	—	—	—
16	Soya bean flour	Protein (N × 6·25) level	8·5	10·6	12·3	15·3
		Soya flour	17·6	22·0	26·4	33·0
		Butter fat	10·0	10·0	10·0	10·0
		Salts*	5·0	5·0	5·0	5·0
		Rice starch	67·4	63·0	58·6	52·0
17	Baker's yeast	Protein (N × 6·25) level	8·4	10·0	12·1	—
		Yeast	17·5	21·9	26·3	—
		Butter fat	10·0	10·0	10·0	—
		Salts*	5·0	5·0	5·0	—
		Rice starch	67·5	63·1	58·7	—

* Steenbock's salt mixture No. 40.

the course of the study, while in Table III the biological values of the proteins are summarised.

Discussion.

Owing to the low nitrogen content several breads have been fed to rats only at one level of protein intake. This level has not necessarily been constant for all the breads studied in the present work. A direct comparison of the biological values of the nitrogen contained in these breads is therefore not possible. While our figures indicate that rye breads in general are superior, as far as the biological value of the protein goes, to wheat products, and that darker rye breads made from less refined flour are in turn superior to lighter varieties, we do not feel entitled, for reasons stated above, to draw definite

Table III.

No.	Diet	Protein (N × 6·25) content %	Sex of rats	Days	Total intake, g. Food*	Total intake, g. Protein*	Initial weight g.	Final weight g.	Gain in weight g.	Gain per g. protein	Average gain per g. protein
1	White wheat bread	8·6	♀	29	229·1	19·7	65	77	12	0·61	0·81
			♂	31	325·5	28·0	85	109	24	0·86	
			♂	30	278·6	24·0	71	94	23	0·96	
		10·4	♀	31	274·7	28·6	70	96	26	0·91	1·09
			♂	31	285·3	29·7	72	103	31	1·04	
			♀	31	274·2	28·5	69	107	38	1·33	
2	Wheat rolls	6·4	♀	31	283·5	18·1	50	68	18	0·99	0·98
			♀	31	307·5	19·7	57	76	19	0·96	
		8·2	♂	31	315·0	25·8	57	86	29	1·12	1·06
			♀	31	318·8	26·1	54	80	26	1·00	
		10·9	♀	31	434·7	47·4	51	103	52	1·10	1·13
			♂	31	409·9	44·7	55	107	52	1·16	
3	White wheat-yeast bread I	9·1	♀	29	261·5	23·8	55	93	38	1·60	1·65
			♂	28	173·7	15·9	42	69	27	1·70	
		10·4	♀	29	327·0	34·0	75	138	63	1·85	1·86
			♀	29	338·9	35·2	74	140	66	1·87	
		12·9	♀	31	288·0	37·3	52	119	67	1·80	1·90
			♂	31	381·0	49·3	54	152	98	1·99	
4	White wheat-yeast bread II	8·3	♀	31	219·7	18·3	52	93	41	2·24	1·87
			♂	31	246·4	20·5	61	92	31	1·51	
		9·6	♀	31	211·6	20·2	54	89	35	1·73	1·60
			♂	31	205·2	19·7	53	82	29	1·47	
		12·3	♀	30	219·6	27·0	48	94	46	1·70	1·91
			♀	31	442·6	54·4	54	170	116	2·13	
5	Soya flour-wheat bread I	8·1	♀	30	248·5	20·1	57	103	46	2·28	1·97
			♀	33	294·8	23·9	71	111	40	1·67	
		10·2	♂	30	238·7	24·4	60	100	40	1·64	1·62
			♀	30	244·4	25·0	65	105	40	1·60	
		11·9	♀	30	229·0	27·3	57	96	39	1·43	1·46
			♂	30	218·7	26·0	57	96	39	1·49	
6	Soya flour-wheat bread II	8·5	♀	30	231·1	19·6	75	102	27	1·37	1·33
			♀	30	264·5	22·5	80	109	29	1·29	
		10·1	♀	30	300·5	30·4	85	138	53	1·74	1·79
			♀	30	322·9	32·6	75	135	60	1·84	
		15·3	♀	30	292·2	44·7	61	152	91	2·03	1·95
			♂	30	265·2	40·6	61	137	76	1·87	
7	70 % rye bread	6·4	♀	31	219·0	14·0	47	63	16	1·14	1·25
			♀	31	227·4	14·6	41	61	20	1·37	
			♀	31	202·4	12·9	45	61	16	1·24	
8	Crust of bread No. 7	6·3	♂	30	236·0	14·9	57	77	20	1·34	0·83
			♂	31	205·5	13·0	65	73	8	0·62	
			♂	31	213·5	13·5	65	72	7	0·52	
9	Crumb of bread No. 7	6·7	♂	31	303·4	20·3	66	95	29	1·42	1·26
			♂	31	278·6	18·7	67	89	22	1·17	
			♂	30	239·3	16·0	64	83	19	1·18	
10	70 % rye bread	6·0	♂	30	272·0	16·3	60	80	20	1·23	1·11
			♀	30	268·0	16·1	56	73	17	1·06	
			♀	30	257·7	15·5	56	72	16	1·03	
11	Rye yeast bread	8·2	♀	31	205·0	16·8	54	67	13	0·77	1·07
			♂	31	330·3	27·1	48	98	50	1·84	
			♂	30	188·5	15·4	59	68	9	0·58	
12	Soya flour-rye bread I	8·0	♀	31	308·9	24·7	55	110	55	2·22	2·04
			♂	31	185·0	14·8	40	62	22	1·50	
			♂	31	203·3	16·3	43	82	39	2·39	
		10·2	♀	31	207·4	21·2	43	75	32	1·51	1·59
			♂	31	314·3	32·1	51	105	54	1·68	

* Water-free basis.

Table III (*contd.*).

No.	Diet	Protein (N ×6·25) content %	Sex of rats	Days	Total intake, g. Food*	Total intake, g. Protein*	Initial weight g.	Final weight g.	Gain in weight g.	Gain per g. protein	Average gain per g. protein
13	Soya flour-rye bread II	8·1	♀	31	286·2	23·2	59	116	57	2·46	2·43
			♀	31	273·0	22·1	55	108	53	2·40	
		10·1	♀	30	231·9	23·4	53	101	48	2·05	1·95
			♀	30	208·4	21·1	48	87	39	1·85	
		11·9	♀	30	237·7	28·3	55	108	53	1·87	2 02
			♀	30	269·7	32·1	53	123	70	2·18	
14	Military bread (rye)	8·6	♀	31	217·2	18·7	44	72	28	1·50	1·75
			♂	31	331·7	28·5	50	103	53	1·85	
			♂	31	431·2	37·1	61	132	71	1·91	
15	Whole-meal country rye bread	9·7	♀	31	268·3	26·0	47	80	33	1·27	1·29
			♂	31	230·8	22·4	42	67	25	1·11	
			♂	31	331·0	32·1	57	105	48	1·49	
16	Soya bean flour	8·5	♀	30	261·8	22·3	61	95	34	1·53	1·64
			♀	30	370·1	31·5	65	120	55	1·75	
		10·6	♂	30	319·2	33·7	74	126	52	1·55	1·57
			♀	30	299·3	31·6	68	118	50	1·59	
		12·3	♂	30	300·8	37·0	72	133	61	1·65	1·51
			♀	29	247·1	30·4	70	112	42	1·38	
		15·3	♂	30	306·1	46·8	53	135	82	1·75	1·58
			♀	30	312·4	47·8	49	119	70	1·46	
			♂	30	316·8	48·5	52	129	77	1·52	
17	Baker's yeast	8·4	♀	30	326·0	27·4	60	100	40	1·46	1·48
			♀	29	273·7	23·0	63	97	34	1·49	
		10·0	♀	30	257·0	25·7	61	99	38	1·48	1·32
			♂	29	287·0	28·7	72	105	33	1·15	
		12·1	♀	30	268·1	32·4	67	107	40	1·23	1·36
			♂	29	300·5	36·4	72	126	54	1·49	

* Water-free basis.

conclusions from the results of these tests. Our data would also seem to indicate that whole-meal rye bread baked from 100 % rye flour is inferior with regard to the biological value of its proteins to dark rye bread prepared from 82 % flour. This might be due to the presence in the former bread of rather large amounts of indigestible roughage derived from the grain husks and interfering with the normal utilisation of the proteins.

While we are thus inclined to present only a series of figures for some of the breads studied without subjecting the former to comparison or discussion, we feel that a more detailed review will be permissible in the case of other breads where biological values obtained at several levels of protein intake are available.

Two wheat-yeast breads, one containing approximately 8 %, the other approximately 12 % of yeast (on the dry basis) have been prepared and tested at three levels of protein intake. Yeast alone was fed at three similar levels, while the two lower levels were used for the study of the biological value of the nitrogen of wheat bread alone. The results are presented in Table III, Nos. 1, 3, 4 and 17. It is evident that in both cases and at all levels of protein intake the combinations of wheat flour and baker's yeast yielded higher biological values than either of the components. It may therefore be concluded that a supplementary relation exists between the proteins of white wheat flour and

those of baker's yeast, when mixed in the approximate proportion of 7 to 1 and 11 to 1.

Owing to technical difficulties in the preparation of rye-yeast breads, only one such bread could be prepared, containing a rather low percentage of yeast (about 4·5 %). When fed at the only level of 8·2 % of protein this bread caused a gain in weight of 1·07 g. per g. of protein ingested, lower than that caused by yeast alone at a corresponding level (8·4 %) and of about the same order as given by rye bread at a 6·0 % level (Table III, Nos. 10, 11 and 17). The results for the rye-yeast bread were very irregular, one rat gaining at a rate of 1·84 g. per g. of protein ingested while two other rats made gains of only 0·77 and 0·58. It is obviously impossible to judge from these results whether or not there exists a supplementary relation between the proteins of rye and those of baker's yeast.

Two soya bean flour-wheat flour breads have been prepared, the ratios of the constituents being 1 to 9 and 1 to 4 respectively. Each of these breads has been fed to rats at three levels of protein intake. Soya bean flour alone has been fed at four levels. As already stated the basal wheat bread was fed at two levels of protein intake (Table III, Nos. 1, 5, 6 and 16). For both soya-wheat breads and at all levels studied the results are better than for wheat bread alone. For the soya bean flour-wheat flour bread I (ratio of constituents 1 to 9) a maximum gain of almost 2 g. per g. of protein ingested has been found at an approximately 8 % level of intake, decidedly superior to the value found at this level for soya bean flour alone and more than double the gain obtained for the basal wheat bread at a similar protein concentration. The second soya-wheat bread (ratio of constituents 1 to 4) produced maximal gains at the highest level of protein intake definitely surpassing those found for soya flour alone at the same level. The basal wheat bread was not fed at this level but it seems exceedingly unlikely that at any plane of protein intake it could produce gains surpassing or equalling the maximum shown by the combination. These results indicate a definite supplementary relation between the proteins of white wheat flour and of soya bean flour when mixed in the approximate proportions of 9 to 1 and 4 to 1.

Two soya bean flour-rye flour breads have been subjected to biological analysis (Table III, Nos. 12 and 13). The ratio of the constituents was the same as in the case of the soya-wheat breads. A basal rye bread was prepared from the same batch of rye flour as used for the combined breads (Table III, No. 10). Owing to the low nitrogen content this bread was fed at only one level of protein intake, soya-rye bread I (ratio of constituents 1 to 9) was tested at two levels while the second combined bread (ratio 1 to 4) was fed at three levels. Maximal values were found for both experimental breads at an 8 % level of protein intake. These values are definitely superior to those found for the proteins of soya bean flour at a similar level. In fact the second experimental bread gave at all three levels of protein intake values superior to those found at corresponding levels for the soya bean nitrogen. As rye bread

alone was not fed at these levels, no direct proof of a supplementary relation between the proteins of the rye flour and of soya bean flour is available. There seems, however, to be little doubt that such a relationship exists, as both the values found for military bread baked from 82 % rye flour and fed at an 8·6 % level and for whole-meal bread (100 % flour) fed at a 9·7 % level and reported in the present work, as well as values found by Osborne and Mendel [1920] for the proteins of the whole rye kernel at 8 and 10 % levels, are definitely lower than the values reported here at corresponding levels. In fact the gain of 2·4 g. per g. of protein ingested produced by the soya-rye combination at an 8 % level of intake is exceptionally good for vegetable proteins.

It will be noted that in the preparation of the experimental breads containing soya bean flour a small amount of foreign nitrogen was necessarily introduced through the addition of yeast (less than 0·5 % of the dry matter) in the process of baking. A supplementary action of this nitrogen cannot be ruled out. We doubt however whether it could have affected the results to any measurable extent at such a low concentration.

In order to verify whether the high temperature to which the crust of bread is exposed in the process of baking has any deterimental influence upon the biological value of the proteins, crumb and crust have been studied separately. Loaves of rye bread obtained from the Municipal Bakeries were split in halves, and the crust separated from the crumb as carefully as possible, first by hand, and then by cautiously cutting away all the caramelised brown layer of crust. Crust, crumb, and the whole bread were studied at the same plane of protein intake (Table III, Nos. 7, 8 and 9) and the biological value of the nitrogen of the crust has been found to be lower than that of the crumb or of the whole bread. There is no difference between whole bread and crumb, showing that probably the injury caused by heat in the proteins of the crust is compensated when they are combined with the proteins of the crumb. In a comprehensive study of the effect of heat on the biological value of proteins Morgan [1931] has recently reported a similar decrease of the biological value of the nitrogen of the crust as compared with the crumb.

It is interesting to note that in the case of soya bean flour a change of the level of protein intake from 8·5 % to 15·3 % caused hardly any difference in the biological value of the protein, making it impossible to select the level giving the maximal gain in weight per g. of protein ingested. The differences are also very slight for wheat rolls (three levels of intake from 6·4 % to 10·9 %) and for baker's yeast (three levels of intake from 8·4 % to 12·1 %). This is in harmony with similar findings of Morgan [1931] in the case of wheat gluten.

SUMMARY.

1. The biological values of the proteins (N × 6·25) of various natural and experimental breads, of baker's yeast and of soya bean flour have been determined by the numerical method of Osborne, Mendel and Ferry.

2. A supplementary relation exists between the proteins of white wheat flour and those of baker's yeast.

3. A supplementary relation also exists between the proteins of white wheat flour and soya bean flour.

4. There is a strong indication that a supplementary relation exists between the proteins of rye flour and of soya bean flour.

5. The biological value of the nitrogen of crust was found to be lower than that of crumb or of whole bread.

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CLXII. THE HEAT-INACTIVATION OF THE PROTEINASE OF THE PANCREAS.

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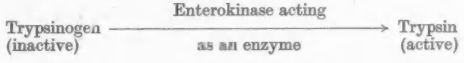
Introduction.

While previous work of the writer, upon the heat inactivation of trypsin, was in progress, it was shown by Waldschmidt-Leitz and Purr [1929] that trypsin itself is not an individual enzyme, but that it may be resolved into two components—a proteinase and a carboxypolypeptidase.

It was found by these authors that the proteinase, although completely inactive alone, is capable, when associated with enterokinase, of hydrolysing proteins and certain of their degradation products. The carboxypolypeptidase, in the absence of enterokinase, attacks a number of polypeptides and some dipeptides, which possess a free carboxyl group in the molecule, but will not attack a protein. Enterokinase increases the activity of carboxypolypeptidase. This work of Waldschmidt-Leitz and Purr has a distinct bearing upon the interrelation of trypsin and enterokinase. It is of interest to recall, very briefly, the views which have been held about this problem.

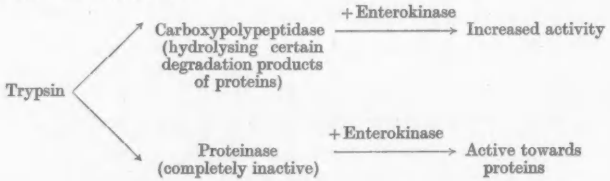
(*a*) *The "classical" view.* It was considered that an inactive zymogen, trypsinogen is produced in the pancreas. The trypsinogen is enzymically transformed into trypsin by enterokinase, and the trypsin thus produced is able to hydrolyse proteins. This may be expressed thus:



(*b*) *The view of the Willstätter school up to 1930.* According to this view the pancreas produces an enzyme, trypsin, which, although able to hydrolyse certain of the products of protein degradation, is inactive towards proteins themselves. Trypsin forms an addition compound with enterokinase, namely, trypsin-kinase, which readily hydrolyses proteins:

Trypsin (inactive towards proteins, but hydrolysing certain degradation products)
+ Enterokinase
Trypsin-kinase (active towards proteins)

(*c*) *The view arising from the work of Waldschmidt-Leitz and Purr.* This may be summarised thus:



Thus proteinase is analogous to the classical trypsinogen.

In the earlier work of the writer [1930] the investigation of the heat-inactivation of trypsin was carried out with solutions containing 24 % glycerol, this circumstance arising from the technique used in preparing the trypsin solutions. Further experiments were planned to study the effect of varying the glycerol concentration so that, by extrapolation, a value of the critical increment of the process in solutions containing no glycerol could be obtained, since such a value would obviously be of importance from the point of view of the behaviour of purely aqueous systems. From the work of Waldschmidt-Leitz and Purr it was apparent, however, that it would be more satisfactory to examine not "trypsin" itself but one of its components, namely proteinase, this component being chosen because it is more closely analogous to the classical trypsinogen (the carboxypolypeptidase component being more analogous to erepsin).

In the method evolved by Waldschmidt-Leitz and Purr for the isolation of the proteinase, we are left, after numerous adsorptions, with this enzyme in solution. Clearly any adjustment of the glycerol concentration would involve still further dilution, with a resulting diminution in concentration of enzyme. In view of this it was evident that as an important preliminary step an attempt should be made to carry the Waldschmidt-Leitz and Purr treatment further by adsorbing proteinase on a suitable adsorbent, say kaolin. The proteinase could then be eluted from the kaolin, always by the same volume of liquid, but the percentage of glycerol in that volume could be previously adjusted. In this way for example the enzyme could be eluted from the kaolin by an aqueous solution, containing no glycerol, and thus the critical increment of the heat inactivation process in water could be determined directly instead of by extrapolation.

Experimental.

(*a*) *The preparation of alumina* C_γ. The alumina used in the isolation of the proteinase was a sample of alumina C prepared according to the technique of Willstätter and Kraut [1923] about 12 months previously and since then kept under distilled water. It was therefore completely transformed into the C_γ modification.

(*b*) *Isolation of the proteinase.* This was carried out in principle according

to the method of Waldschmidt-Leitz and Purr, the actual procedure adopted being as follows. 100 cc. of ice-cold glycerol extract of dried pancreas were brought to p_H 4·0 by addition of 75 cc. of ice-cold buffer solution. (The buffer solution was made up by mixing 17·6 cc. *N* acetic acid and 2·04 cc. *N* sodium acetate and diluting to 100 cc.). 5 cc. of alumina C_γ suspension (containing 105 mg. Al_2O_3) were then added to the buffered enzyme extract. The mixture was shaken, allowed to stand in the ice-chest for about 10 minutes and then centrifuged. The clear supernatant liquid was then treated in exactly the same way with a second 5 cc. of alumina C_γ suspension. This operation, which was repeated four times in all, removes the dipeptidase and aminopolypeptidase. The clear solution from the final adsorption was then brought to p_H 7·0 by addition of *N* ammonia. To the solution, now at p_H 7·0, were added 5 cc. of alumina C_γ suspension. The mixture was shaken, allowed to stand in the ice-chest for about 10 minutes and then centrifuged. In all ten such adsorptions at p_H 7·0 were carried out upon the solution. This procedure removes the carboxypolypeptidase, and the solution from the final adsorption contained the proteinase.

This solution, which was used in the later experiments with kaolin, was kept in the ice-chest. Prior to treatment with kaolin, the proteinase content of the solution was determined by activating with enterokinase and measuring the resulting activity according to the method of Linderstrøm-Lang and Steenberg [1929]. It was found that the solution contained 0·95 enzyme unit per cc.

(*c*) *Adsorption of the proteinase upon kaolin.* Experiments were next carried out to determine whether the proteinase is adsorbed by kaolin, since in the experiments of Willstätter *et al.* [1926] it was found that "trypsin," under certain conditions, is adsorbed by kaolin.

To 30 cc. of the ice-cold proteinase solution at p_H 7·0 were added 5 cc. of a suspension of kaolin in distilled water, which contained 0·4 g. of B.D.H. kaolin *puriss.* The mixture was shaken and left for 10 minutes in the ice-chest, after which it was centrifuged. The clear centrifugate was treated again with 5 cc. of kaolin suspension and the above procedure repeated. This operation was carried out four times in all.

The next process was to attempt the elution of the proteinase from the kaolin and thus examine the degree of success obtained with the adsorption process. Accordingly the kaolin from the adsorption experiments was shaken up with 30 cc. of ice-cold solution E. (Solution E was made up of 57 parts by volume of 1 % diammonium phosphate solution, 3 parts by volume of *N* ammonia, and 40 parts by volume of glycerol-water solution—this solution having already been employed by Willstätter *et al.* [1926] in connection with trypsin.) The suspension of kaolin in solution E was kept in the ice-chest for 15 minutes and was then centrifuged. The clear liquid from the centrifuging was then neutralised with *N* acetic acid and kept in the ice-chest. The proteinase content of this solution was then examined, and it was found to be

0·78 enzyme unit per cc. That is, the process of adsorption on kaolin together with the subsequent elution may be considered quite satisfactory.

The heat-inactivation of the proteinase.

For the experiments in this section the starting material was the stock solution of proteinase remaining after the alumina adsorptions. Immediately before a run 30 cc. of this solution were treated with kaolin and the proteinase then eluted from the kaolin as described above. By varying the glycerol concentration in the solution employed for the elution, it was possible to examine the heat-inactivation of the proteinase in solutions differing in glycerol content, but identical in electrolyte concentration.

In the first place it was necessary to examine whether the heat-inactivation of the proteinase resembled that of "trypsin" in respect of the applicability of the unimolecular expression. The p_H measurements in this work were made by means of the glass electrode at room temperature. By way of illustration Table I gives the data obtained in aqueous solution.

Table I.

Temperature 50°. Glycerol 0 %. p_H 6·8.

Time (mins.)	Enzyme units per cc.	$k \times 10^3$
0	0·78	—
45	0·57	7·0
90	0·42	6·9
135	0·30	7·1

It is seen that there is agreement with the unimolecular expression. With solutions containing glycerol agreement with the unimolecular expression was also found.

The critical increment of the process at different glycerol concentrations.

The critical increment of the process was determined by measuring the value of k at 50° and at 60° and then substituting into the integrated form of the equation:

$$\frac{d \ln k}{dT} = \frac{E}{RT^2}.$$

The experiments were carried out with solutions containing 40, 20 and 0 % glycerol respectively. It was not feasible to work with a glycerol concentration greater than 40 %, because of the extreme difficulty in centrifuging the kaolin in solutions of higher glycerol content.

The results obtained are given in Table II.

Thus it is seen that the magnitude of the critical increment is sensibly independent of the glycerol concentration, lying between 35,000 and 40,000 calories per "molar unit" of enzyme.

Table II.

		Time (mins.)	Enzyme units per cc.	k	E (calories)
Glycerol 0 % p_H 6·9	Temp. 50°	0	0·80	$7·6 \times 10^{-3}$	39,000
		70	0·47		
	Temp. 60°	0	0·80	$4·7 \times 10^{-2}$	
		10	0·50		
Glycerol 20 % p_H 6·8	Temp. 50°	0	0·73	$3·6 \times 10^{-3}$	40,000
		90	0·53		
	Temp. 60°	0	0·72	$2·3 \times 10^{-2}$	
		15	0·51		
Glycerol 40 % p_H 6·9	Temp. 50°	0	0·76	$2·1 \times 10^{-3}$	35,000
		110	0·60		
	Temp. 60°	0	0·78	$1·1 \times 10^{-2}$	
		15	0·66		

Discussion.

From the results given above we may make comparisons, especially in relation to the results of other work carried out in this laboratory, which may be tabulated as follows.

1. The magnitude of the critical increment for heat-inactivation of the proteinase is practically identical with that found for trypsin-kinase, enterokinase and "trypsin." Thus the separation of the carboxypolypeptidase has not influenced quantitatively the thermal stability of the proteinase.

2. The critical increment, within the limits of experimental error, is independent of the glycerol concentration of the solution, and we may conclude by inference that this holds true for "trypsin."

3. The fact that the critical increment does not vary with the glycerol concentration is in agreement with the similar result found by McGillivray [1930] for purified lipase.

4. The magnitude of the critical increment approximates to that found by McGillivray for purified lipase, namely 46,000 calories.

Probably the most striking of these facts is the resemblance between the behaviour of proteinase, trypsin-kinase, enterokinase and that of lipase. Such a resemblance between enzymes differing so markedly in specificity as do lipase and proteinase indicates that, although heat-inactivation results in destruction of their specific active groupings, the primary effect must be upon the supporting "core" of each enzyme. Further, it seems reasonable to conclude that the cores of each enzyme must be very closely similar, either in chemical structure or in the physical state of their aggregation. Their difference in specificity may be governed by the spatial distribution of active groups on the surface of the core.

In general it will be observed that the values obtained by the writer and by McGillivray for the critical increment are less than those which have been found by previous workers, who have for the most part employed cruder enzyme preparations. McGillivray showed that the purification of lipase is accompanied by a drop in the value of the critical increment of heat-inactivation from 95,000 calories to 46,000 calories. Yet it might still be considered

that the magnitude, *circa* 40,000 calories, found for the proteinase and lipase is still influenced by the presence of adventitious material associated with the true core of the enzyme. It is clear, however, by considering the case of the proteinase, that such material is so firmly attached to the enzyme that it defies separation therefrom by methods of adsorption which have been successfully applied to separate enzymes which must be regarded as very analogous in their adsorption affinities. Thus such material, if present, may very well be regarded as an integral part of the enzyme core.

Summary.

1. Experiments have been carried out upon the adsorption by means of kaolin of proteinase obtained in solution by the method of Waldschmidt-Leitz and Purr [1929]. It is found that the proteinase is removed from solution by the kaolin and may be successfully eluted therefrom. This additional procedure has been incorporated with the technique of Waldschmidt-Leitz and Purr to prepare solutions of the proteinase.

2. It is found that the heat-inactivation of the proteinase proceeds in agreement with the unimolecular expression.

3. The critical increment of heat-inactivation has been determined in solutions containing 40, 20 and 0 % glycerol. It is found that the critical increment is independent of the glycerol concentration, and is of the value of 35,000–40,000 calories per molar unit of enzyme.

4. Resemblances between the heat-inactivation of proteinase, "trypsin," enterokinase and purified lipase are discussed. It is suggested that in general heat-inactivation is primarily an effect upon the enzyme core. It is pointed out in addition that it seems reasonable to conclude that different enzymes must possess cores which are closely similar, either in chemical structure or in the physical state of their aggregation.

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CLXIII. AUTOLYSIS IN MALIGNANT AND NORMAL RABBIT TISSUES[1].

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In connection with proteolytic change in malignant growths Robin [1919] arrived at the following conclusions, based upon an analysis of malignant and normal human tissue.

(1) There is "a decrease in the insoluble nitrogen and an increase in the soluble nitrogen in the 'relatively healthy' regions bordering upon the growth as compared not only with the most cancerous regions but also with normal healthy tissue."

(2) There is "an increase in the insoluble nitrogen, *i.e.* protein nitrogen, in the parts of the tissue which are most attacked, with a diminution in soluble nitrogen."

In other words the percentage ratio of protein nitrogen to total nitrogen is lower in the "relatively healthy" region than in either the growth or the normal tissue. A typical example of Robin's findings is as follows:

	$\frac{\text{Protein N}}{\text{Total N}} \times 100$
Most cancerous region	58·01
Relatively healthy region	47·01
Normal liver (after 26 hours' autolysis)	70·55

Robin considered that the most logical interpretation of these facts was that the proteins of a cancer are formed at the expense of the products of autolysis of the proteins of the organ in which it develops. This view implies that the tissue immediately surrounding the growth is abnormal with respect to autolysis, and the point is of such importance that it was considered worthy of further investigation.

In the present investigation nitrogen analyses were carried out using rabbit tissues—cancerous, adjacent normal tissue, and the normal tissue of the same organ.

Experimental.

The rabbit tissue used for the experiments was liver and kidney in which metastatic deposits of malignant growth were present, the rabbits having been

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation, Prof. W. Blair Bell, Director, University of Liverpool.

previously inoculated with the Brown-Pearce tumour. On removing the organ from the animal the growth was cut away from the rest of the organ and the adjacent normal tissue also removed from the rest of the normal tissue. A representative sample of each section was submitted to independent histological examination.

The tissue was first minced up as finely as possible with a pair of scissors and then weighed out in portions of 0·3–0·5 g. This is the smallest weight of tissue compatible with uniform samples. Six samples of each section of tissue were weighed out, two in combustion test-tubes for total nitrogen determinations, two in small glass tubes for protein nitrogen determinations and two in small tubes for dry weight estimations. The last two were heated at 105–115° for 36 hours in an electric oven. It was found that this drying was enough to give constant dry weights and the results could be reproduced to within 2 %.

Following the well-known micro-Kjeldahl technique [Pregl, 1924] for total nitrogen determination, there was added to each sample 1 cc. concentrated sulphuric acid, a knife-point of nitrogen-free copper sulphate and the same amount of potassium sulphate, and the mixture heated over a micro-burner until it charred. A few drops of 30 % hydrogen peroxide were then added and the mixture again heated to charring. This process was repeated until all the organic matter had disappeared, judging by the absence of charring. The time required is shortened considerably if before adding the sulphates the tissue is charred with concentrated sulphuric acid then heated for some time in a water-bath, a few drops of hydrogen peroxide being added from time to time. After the oxidation was complete the contents of the tube were diluted and made up to 25 cc. 2 cc. samples were taken and the nitrogen estimated in a micro-Kjeldahl distillation apparatus. Blank experiments were made with concentrated sulphuric acid, copper sulphate, potassium sulphate, distilled water and hydrogen peroxide.

Each sample for protein determination was thoroughly mixed with pure anhydrous sodium sulphate and allowed to harden. The hard mass was transferred to a small flask with absolute alcohol and boiled on a water-bath for an hour. The liquid was then centrifuged and the remaining mass extracted with boiling water slightly acidified with acetic acid. The extraction was continued until the extracting liquid showed no sign of sulphate. The remaining proteins were incinerated and the nitrogen estimated by the micro-Kjeldahl method as before.

Samples (in duplicate) were taken from tissue representing the three regions, *viz.* the growth, the adjacent normal tissue, and the normal tissue, and also duplicate distillations were carried out with each sample. The results obtained by the above method agree to within 2 %.

Results.

In all, tissues from 16 rabbits were analysed and the results are summarised in Table I.

Table I. *Analytical results.*

Rabbit No.		6	7	11	12	13	4	5	8	2	1	10	14	15	16	3	9
Description of tissue		K.	L.	L.	L.	K.	K.	L.	L.	K.	K.	K.	K.	L.	K.	K.	K.
P.M. period (hours)		0	0	0	0	0	1	1	3	5	12	12	12	12	12	24	24
Growth	Dry weight	18·9	21·4	21·8	22·3	20·4	15·5	22·7	21·3	21·2	20·5	18·1	22·0	24·8	20·6	20·3	20·9
	Total nitrogen	2·68	2·86	2·79	3·05	2·79	2·83	3·36	2·92	3·26	2·75	2·13	3·26	3·67	2·84	2·70	2·69
	Protein ,,	1·72	2·42	2·44	2·55	2·21	2·26	2·56	2·35	2·63	2·34	1·97	2·62	3·23	2·60	2·45	2·23
	Soluble ,,	0·96	0·44	0·35	0·50	0·58	0·57	0·80	0·57	0·63	0·41	0·16	0·64	0·44	0·24	0·25	0·46
Adjacent normal tissue	Dry weight	20·4	24·0	23·5	26·7	22·2	16·6	22·2	23·0	19·8	20·2	19·6	20·5	24·1	19·6	17·5	17·4
	Total nitrogen	3·03	3·25	2·98	3·48	3·66	2·27	3·17	3·39	3·15	2·72	2·59	2·88	3·76	2·78	2·38	2·44
	Protein ,,	2·27	2·90	2·70	3·00	3·24	1·79	2·45	2·89	2·45	2·02	2·22	2·20	2·97	2·30	2·10	1·96
	Soluble ,,	0·76	0·35	0·28	0·48	0·42	0·48	0·72	0·50	0·70	0·70	0·37	0·68	0·79	0·48	0·28	0·48
Normal tissue	Dry weight	19·9	—	22·6	25·2	21·8	15·9	23·5	25·1	18·6	—	15·9	22·9	24·2	18·2	15·9	17·9
	Total nitrogen	2·74	—	2·56	3·73	3·44	2·25	3·08	3·51	2·81	2·80	1·96	2·85	3·68	2·30	2·14	2·41
	Protein ,,	2·13	—	2·48	3·61	3·20	1·76	2·70	3·09	2·35	2·62	1·83	2·53	2·92	2·15	1·91	2·05
	Soluble ,,	0·61	—	0·08	0·12	0·20	0·49	0·38	0·50	0·46	0·18	0·13	0·32	0·39	0·15	0·23	0·36

Percentage ratios of protein nitrogen to total nitrogen

P.M. period	0	0	0	0	0	1	1	3	5	12	12	12	12	12	24	24
Growth	64·2	84·6	87·5	83·6	79·2	79·9	76·2	80·5	80·7	85·1	92·5	80·4	88·0	91·6	90·7	82·9
Adjacent normal tissue	67·3	89·2	90·6	86·2	88·5	78·9	77·3	85·3	77·8	74·3	85·7	76·4	79·0	82·7	88·2	80·3
Normal tissue	77·7	—	96·9	96·7	93·1	78·2	87·7	88·0	83·6	93·6	93·4	88·8	79·4	93·5	89·3	85·1

In the second horizontal column the letters K. and L. represent kidney and liver respectively.

The *post mortem* period, represented by P.M. period, is the time elapsing between the death of the animal and the excision of the tissue.

The "dry weight" is the percentage ratio of the weight of dried tissue to the weight of the tissue before drying, *i.e.* fresh tissue.

The nitrogen figures represent, in all cases, the percentage weight of nitrogen in fresh, *i.e.* wet tissue.

At the foot of Table I are given the percentage ratios of protein nitrogen to total nitrogen in the growth, adjacent normal tissue and normal tissue.

Discussion of results.

In the first place it is necessary to draw attention to the conditions under which Robin's results were obtained. Robin worked with human liver, stomach and pancreas which were obtained *post mortem*. As a consequence the tissue was not excised from the corpse until 24–27 hours after death. As will be seen later this fact probably accounts for the results that Robin obtained. The normal tissue employed by Robin was tissue from a healthy person killed in an accident. In the present investigation the normal tissue was taken from the same organ as the growth and consequently probably gives a more useful comparison than that of Robin. It follows that Robin's results and the group with P.M. period of 12 hours in the present investigation ought to be comparable.

The results obtained by the writer indicate fundamental differences according to the P.M. period of the tissue employed. In the group with P.M. period less than 3 hours the percentage ratio of protein nitrogen to total nitrogen is greatest in the normal tissue, lowest in the growth, with an intermediate value in the adjacent normal tissue. When the P.M. period is 12 hours the ratio is lower in the adjacent normal tissue than in either the normal tissue or the growth—this might for convenience be known as the "Robin effect." When the P.M. period exceeds 12 hours this "Robin effect" seems to be less pronounced. Hence if the tissue is analysed when it is as near as possible to the condition obtaining *in vivo*, *i.e.* P.M. period less than 3 hours, there is no unexpected proteolytic condition in the adjacent normal tissue. The "Robin effect" manifests itself after a *post mortem* period of 12 hours for rabbit tissue. Hence this effect is probably due to *post mortem* changes and does not represent conditions of malignancy *in vivo*.

At first sight this minimum value of the ratio protein nitrogen to total nitrogen in the adjacent normal tissue after a P.M. period of 12 hours seems to be explained by assuming that autolysis takes place to the greatest extent in this region. This can be shown not to be the case by consideration of Table II. The second column gives the percentage values of protein nitrogen in the adjacent normal tissue, and the third column gives the percentage

values of protein nitrogen in the normal tissue calculated on the basis of the same dry weight as the corresponding adjacent normal tissue.

Table II.

Rabbit No.	Protein N of the adjacent normal tissue	Calc. protein N of the normal tissue	Rabbit No.	Protein N of the adjacent normal tissue	Calc. protein N of the normal tissue
2	2·45	2·50	10	2·22	2·26
3	2·10	2·10	11	2·70	2·58
4	1·79	1·84	12	(3·00)	(3·72)
5	2·45	2·55	13	3·24	3·21
6	2·27	2·18	14	2·20	2·26
8	2·89	2·83	15	2·97	2·91
9	1·96	1·99	16	2·30	2·32

It will be seen that in each case, whatever the P.M. period, the values in columns 2 and 3 are the same within experimental error. This shows that autolysis is not occurring at an excessive rate in the adjacent normal tissue. Incidentally the above figures show that the growth does not, *in vivo* or *post mortem*, liberate proteolytic enzymes into the adjacent tissue.

In an attempt to understand these *post mortem* changes the figures giving the concentration of soluble nitrogen in the tissues may also with advantage be classified according to the P.M. period of the tissue:

Table III. *Concentration of soluble nitrogen.*

(% N per weight of water in tissue.)

	Rabbit No.	P.M. period	Growth	Adjacent normal tissue	Normal tissue
Kidney	4	1	0·68	0·58	0·58
,,	6	0	1·18	0·96	0·76
,,	13	0	0·73	0·54	0·26
Liver	5	1	1·04	0·93	0·49
,,	7	0	0·56	0·46	—
,,	8	3	0·72	0·65	0·67
,,	11	0	0·45	0·37	0·10
,,	12	0	0·64	0·66	0·16
Kidney	2	5	0·80	0·87	0·57
,,	1	12	0·52	0·88	—
,,	10	12	0·20	0·46	0·16
,,	14	12	0·82	0·86	0·42
,,	16	12	0·15	0·48	0·24
Liver	15	12	0·59	1·04	1·00
Kidney	9	24	0·58	0·58	0·43
,,	3	24	0·30	0·34	0·27

It will be noticed that when the P.M. period is less than 3 hours the concentration of soluble nitrogen in the growth tends to be greater than that in the adjacent normal tissue which in turn tends to be greater than in the normal tissue.

When the P.M. period is 12 hours the concentration of soluble nitrogen seems to reach a maximum in the adjacent normal tissue. This is also the case in Robin's experiments on human tissue 24–27 hours *post mortem*.

When the P.M. period is 24 hours there seems to be a tendency for the concentration of soluble nitrogen to be equal in all three tissue regions.

From these figures it might be assumed that *in vivo* the concentration of soluble nitrogen is highest in the growth, becoming lower in the adjacent tissue and lower still in tissue further away from the growth. Hence on death it is possible that some controlling factor, such as the degree of permeability of the membranes, is altered and the soluble nitrogen travels from the place of highest osmotic pressure, *i.e.* the growth, to lower osmotic pressure regions. Thus in process of time soluble nitrogen will be transferred from the growth in the first instance to the adjacent normal tissue. This will mean that in the growth the total nitrogen will be lower while the protein nitrogen remains the same, that is the ratio of protein nitrogen to total nitrogen will be higher than *in vivo*. Also the adjacent normal tissue will increase in total nitrogen while the protein nitrogen is unaltered, so that the ratio of protein nitrogen to total nitrogen will be lower than *in vivo*. This is the condition we have called the "Robin effect."

The maximum concentration of soluble nitrogen in the adjacent normal tissue after 12 hours *post mortem* is rather puzzling but it is possibly due to some property of the tissue membranes. After 24 hours the membranes may be regarded as having become completely permeable and the concentration of soluble nitrogen tends to be the same in each of the regions.

In Table I all the percentages are referred to fresh (wet) tissue. Additional evidence in support of *post mortem* diffusion causing the different effects is however afforded by an examination of the dry weights of the tissues. From Table IV it will be noticed that when the P.M. period is less than 3 hours the

Table IV. *Dry weights.*

	Rabbit No.	P.M. period	Normal tissue	Growth
Kidney	4	1	15·9	15·5
,,	6	0	19·9	15·5
,,	13	0	21·8	20·4
Liver	5	1	23·5	22·7
,,	7	0	—	21·1
,,	8	3	25·1	21·3
,,	11	0	22·6	21·8
,,	12	0	25·2	22·3
Kidney	2	5	18·6	21·2
,,	10	12	15·9	18·1
,,	1	12	—	20·5
,,	14	12	22·9	22·0
,,	16	12	18·2	20·6
Liver	15	12	24·2	24·8
Kidney	9	24	17·9	20·9
,,	3	24	15·9	20·3

growth tends to contain more water than the normal tissue. This is the usual behaviour of rapidly growing tissues. However when the P.M. period is 12 hours and over, the position is reversed and the normal tissue tends to be greater in water content than the malignant region. Hence, after death, water

begins to disappear from the growth into the surrounding organ. This movement of water makes it very probable that soluble nitrogen is also transferred throughout the organ after death.

The evidence against the Robin view that the normal tissue adjacent to a malignant growth is abnormal *in vivo* in a proteolytic sense is the following:

(*a*) the histological examination of normal tissue and adjacent normal tissue does not indicate any difference between them;

(*b*) in both regions the protein nitrogen content is the same when calculated on the same basis with respect to water content;

(*c*) when the P.M. period is less than 3 hours the ratio of protein to total nitrogen in the adjacent normal tissue is intermediate between the values for the growth and the normal tissue and not below both of them.

SUMMARY.

Malignant, adjacent normal, and normal rabbit tissues have been analysed for total nitrogen, protein nitrogen and soluble nitrogen. The results obtained show that Robin's [1919] view that in malignancy, normal tissue is autolysed and that the growth builds up its proteins from the autolytic products is not correct. The "Robin effect" has been obtained by allowing the tissue to remain in the body for 12 hours after death before excision. This effect has been shown to be due probably to *post mortem* diffusion in the organ. The "Robin effect" does not manifest itself in the case of freshly examined tissue and is therefore not operative *in vivo*.

Evidençe has been obtained to the effect that after death water disappears from the growth into the surrounding tissue.

Evidence has also been obtained that a malignant growth does not liberate proteolytic enzymes into the surrounding tissues.

The writer wishes to acknowledge his indebtedness to Dr M. M. Datnow for the supply of rabbit tissues and also for the histological examination of the tissues.

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CLXIV. THE ACID-DENATURATION OF HAEMOGLOBIN.

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The change undergone by proteins in aqueous solutions when subjected to the action of various influences, such as those of acid, alkali, heat, light and alcohol, is known as "denaturation." Acids and alkalis produce metaprotein, a product soluble in acids and alkalis, but, in the absence of salts, insoluble at the isoelectric point. Boiling an isoelectric suspension of metaprotein converts it into an insoluble product like that obtained from native isoelectric protein under the influence of heat, alcohol, or shaking.

The coagulation of haemoglobin has been studied by Chick and Martin [1910], who used heat as denaturant: they found denaturation to take place only in the presence of water, and to follow the unimolecular law. They found the reaction to be characterised by the temperature coefficient of 1·3 per 1°, a value confirmed by Hartridge [1912], and by Lewis [1926]. Lewis obtained evidence that not only did H^+ and OH^- ions catalyse the reaction, but that water also entered into it, as a reactant rather than as a catalyst. He confirmed the observation of Chick and Martin that under certain specified conditions salts decrease the rate of denaturation, and his work was extended by Cubin [1929], who found that formaldehyde prevented the flocculation of denatured haemoglobin at p_H values greater than 7.

Wu [1929] distinguishes between "denatured" and "coagulated" protein. He regards them as chemical species distinct from each other. "Coagulation" occurs when protein is heated at the isoelectric point or when it is subjected to dehydrating agents, and involves a decrease in the number of free amino- and carboxyl-groups. Such condensed protein is rather insoluble in acids and alkalis. "Denatured" protein is obtained by the action of acids and alkalis, especially on warming, and denaturation consists, according to Wu, in the splitting of the protein with liberation of free $-NH_2$ and $-COOH$ groups. Such denatured protein is much more soluble in acids and bases than is the condensed material.

The action of alcohol upon methaemoglobin is to give a substance which has lost the power of combining with oxygen [Arnold, 1900]. It was studied by Van Klaveren [1901] under the name of kathaemoglobin, and is now called

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parahaematin [Keilin, 1926]. Alkali yields a similar product [Adair, unpublished observation quoted by Keilin, 1926], as do many other reagents, and it is now clear that parahaematin is a compound between denatured, or condensed, protein and oxidised haematin [Keilin, 1926]. Reduction gives the haemochromogen of Bertin-Sans and de Moitessier [1893] and of Anson and Mirsky [1925].

In general the denaturation of proteins has been regarded as irreversible. Though Michaelis and Rona [1910] report the heat-coagulation of electrolyte-free serum-albumin to be reversed by the action of alkalis, an observation confirmed by Spiegel-Adolf [1926], no like case appeared to be known, either with regard to condensed or denatured proteins, until Anson and Mirsky [1925] stated that the acid-denaturation of haemoglobin was essentially reversible. When a solution of haemoglobin was acidified and the mixture neutralised, complete precipitation resulted. By the action of alkalis on this coagulum, especially in the presence of cyanide, native haemoglobin could be isolated, best as the carbon monoxide derivative. It may be urged that some native material is carried down by the denatured protein on flocculating. Anson and Mirsky advanced two arguments against this: the yield of denatured reversed protein was about 70 %—yet when entirely denatured protein was precipitated in the presence of native, the latter was undiminished in concentration. Secondly the temperature of denaturation had no effect on the yield of reversed material obtained.

The problem has a dual nature. In the first place haemoglobin behaves as a typical protein, denatured or native. If its denaturation be reversible then it appears probable that the denaturation of other proteins may be so likewise. In the second place this phenomenon has bearings on the haemoglobin system itself. Anson and Mirsky [1925] regard haemoglobin and haemochromogen as forming an equilibrium, the point of balance being decided by the p_H. At strongly acid p_H values haemochromogen predominates in the equilibrium,

$$\text{haemoglobin} \rightleftharpoons \text{haemochromogen},$$

while near neutrality the protein is mainly haemoglobin.

In later experiments they have shown [1930] that the prosthetic group plays no decisive part in affecting reversibility. It is possible to cause the reversion of the denaturation of free globin in the complete absence of haematin. Yet the haematin moiety does play some part in the phenomena observed. Thus Wu and Lin [1927] observed that the amount of reversed protein was greater in the ratio of 1·8 to 1 when the initial protein was reduced haemoglobin in place of oxyhaemoglobin. The haemoglobins of various species appear to behave differently. When a solution of ox haemoglobin is acidified with acid, in the ratio of 4 cc. $N/5$ HCl to 6 cc. 10 % haemoglobin, and neutralised after at least 10 minutes, complete precipitation results, the colour of the coagulum being brown like that of acid haematin. After standing for a few minutes the colour changes to the red of parahaematin, and methaemo-

globin passes into solution. With horse haemoglobin the effects are different. The brown colour of the coagulum changes to the pink of parahaematin, but solution of methaemoglobin does not take place so readily, and the alkaline cyanide reagent of Anson and Mirsky must be used for native material to be obtained in the soluble state. The parahaematin of the haemoglobin of the horse seems to be stable over a wider p_H range than that of the ox (personal communication of Mr R. Hill).

Experimental.

When a solution of oxyhaemoglobin is acidified, changes take place in the system: acid methaemoglobin is formed if the p_H be not too low, and the solution contains free native and free denatured globin, as well as acid haematin. The analysis of such a system is complex; the first phenomenon studied was the formation of acid haematin from acid methaemoglobin.

In the majority of these experiments a solution of methaemoglobin was used. Ox blood corpuscles were washed with isotonic saline four times, laked with an equal volume of distilled water, and the stromata removed with alumina cream. Thereafter the procedure was identical with that advocated by Stadie and Ross [1926] for the electrodialysis of haemoglobin, except that the protein was first converted with 10 % ferricyanide to methaemoglobin.

The buffers employed throughout are those of McIlvaine (0·1 *M* citric acid and 0·2 *M* alkaline phosphate). The buffering power of these buffers is approximately linear throughout the p_H range used here, from p_H 2·2 to 4·8.

A titration curve of each methaemoglobin solution used was constructed by adding HCl in known amount and determining the p_H by the quinhydrone electrode. At the p_H values concerned this does not appear to denature the proteins. Thus the p_H values were not appreciably affected on allowing the solutions to stand with quinhydrone as they would have been had denaturation occurred progressively. A similar titration curve was obtained for gum arabic in 8 % solution. From these curves the amount of acid needed to bring the protein and gum arabic solutions to any p_H could be calculated roughly.

The experiments were carried out as follows. To buffer solution in a standard flask was added the solution of methaemoglobin to be investigated. The buffering power of the protein was overcome by adding acid in the amount calculated from the titration curves referred to. The final p_H was thus that of the buffer. It was found to be a matter of indifference here whether the acid was run into the solution before, or after, addition of the protein solution. The acid solution of protein thus obtained then contained acid methaemoglobin and acid haematin. The relative amounts of these substances were then estimated. This was done by use of the double-wedge trough described by Hill [1929].

In these experiments on acid methaemoglobin and acid haematin, the standard solutions put in the trough, one in each side, consisted of acid

methaemoglobin at p_H about 5·8[1], and acid haematin in 4 % gum arabic solution, dissolved in McIlvaine buffer at the p_H of the acid mixture of methaemoglobin and acid haematin to be analysed. Ten or twenty readings were then taken at the positions where the spectrum of the standards matched in all respects that of the solution analysed. Two sources of error militate against accuracy. The degree of dispersion affects the absorption [Keilin, 1926], and the standard contains no parahaematin. The latter effect can be of small importance, as the absorption of parahaematin is slight, and as it can only be in low concentration. In studying the formation of acid haematin from acid methaemoglobin the author has accepted the view of Keilin that the former indicates a physical state, namely a colloidal suspension of haemin protected by protein or gum arabic, rather than a definite chemical individual.

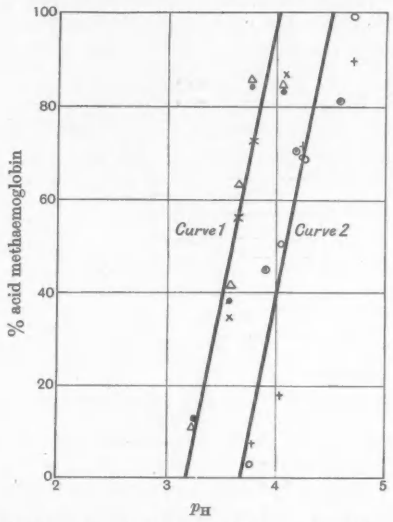

Fig. 1. The formation of acid haematin from acid methaemoglobin.

Curve 1. 2 cc. (approx.) 5 % solution of electrodialysed ox methaemoglobin + 12·5 cc. of McIlvaine buffer + 12·5 cc. water. 0·1 *N* HCl was then added, in amount sufficient to overcome the buffering of the protein and the volume diluted to 50 cc. The standards were acid methaemoglobin at a p_H of 5·5 in water, and a solution of acid haematin in 4 % gum arabic and McIlvaine buffer (12·5 cc. in 50 cc. total volume) at the p_H of the solution studied.

Each different type of point ×, Δ, and ●, refers to separate experiments on the protein.

Curve 2 was carried out with a different sample of protein.

Points + were obtained with 40 cc. of McIlvaine buffer in 50 cc. aqueous solution, ○ points with 20 cc. buffer in 50 cc. total volume. A third sample of protein has been studied. Its behaviour is shown by points ⊙. The experiments were carried out in McIlvaine buffer undiluted.

Curves 1 and 2 (Fig. 1) show formation of acid haematin from acid methaemoglobin as a function of p_H. Two different protein solutions have been studied. The results are similar but the curves do not coincide. On the

[1] It is not possible to have the methaemoglobin standard at the p_H of that to be analysed, for under such conditions it would dissociate to acid haematin and become denatured.

other hand, separate estimations on any one protein preparation agreed admirably, as can be seen from Fig. 1. Solutions of methaemoglobin brought to a p_H of 3·3 or 3·5 in McIlvaine buffer precipitate completely, as though fully denatured, when made isoelectric.

The protein solutions studied in curves 1 and 2 were treated with the alkaline cyanide reagent of Anson and Mirsky, and allowed to stand at a p_H of about 7·8 for 3 hours. Then the solutions were half saturated with ammonium sulphate and the precipitated protein filtered off. On reducing with sodium hydrosulphite and passing in carbon monoxide, the spectrum of carboxyhaemoglobin became visible. The whole was filtered after standing overnight. The total amount of the "reversed" protein was then estimated in the double-wedge trough. The standard solutions were carboxyhaemoglobin in one wedge and water in the other. The yields of native reversed protein obtained from the protein solutions which gave Fig. 1 are plotted in Fig. 2. It seems, that though there appears to be some acid methaemoglobin in

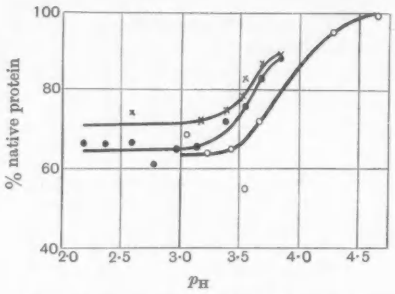

Fig. 2. Amount of carboxyhaemoglobin obtained from acid protein after the treatment with alkaline cyanide advocated by Anson and Mirsky.

The points have the same meaning as in Fig. 1.

solutions which precipitate entirely on neutralisation, yet this acid methaemoglobin is insufficient in amount to account for the yield of "reversed" protein obtained. This discrepancy is tabulated in Table I. It is perhaps due to the dissociation to acid haematin and free native globin. As the properties of protein solutions change rapidly with time, it is a matter of some difficulty to obtain sufficient data in the course of a few days from one solution of protein to provide a comparison of the amounts of acid methaemoglobin present and reversed protein obtained.

The behaviour of acid protein on neutralisation is worthy of closer examination. When a solution of methaemoglobin is brought to p_H 2·5, say, and then neutralised, all the protein is precipitated, and on redissolving the coagulum in the minimum amount of alkali and neutralising the alkaline solution as rapidly as possible, all the protein is again reprecipitated, and on filtering the coagulum only a small fraction of the total protein comes with the filtrate.

Table I.

p_H	Acid methaemoglobin estimated in double-wedge trough: Exp. 1 %	Exp. 2 %	Exp. 3 %	Native protein estimated in double-wedge trough as carboxyhaemoglobin: Exp. 2 %	Exp. 3 %	Value of R: Exp. 2 %	Exp. 3 %
			Protein preparation No. 1				
4·57	91·5	93·8	92	86·7 (?)	—	0·0	0·0 (?)
4·03	85	84	86	82	88·3	4·1	14
3·81	86	85·7	71·9	88	88·3	29·7	29·1
3·66	65·3	30·4 (?)	56·9	83·4	89·1	44·9	34·9
3·56	43·2	38·5	35	73·1	86·6	53·2	60
3·32	12·2	12·6	11·4	73	79·8	83·5	84·5
3·19	0	0	0	64·8	77·2	—	—
2·94	0	0	0	64·2	76·3	—	—
2·76	0	0	0	60·7	—	—	—
2·60	0	0	0	64·6	74·9	—	—
2·43	0	0	0	66	76·6	—	—
2·21	0	0	0	66	77·2	—	—
			Protein preparation No. 2			Mean values of R given here	
4·62	88·6	100·0	—	100	—	0·0 %	
4·24	71·7	69·1	—	95	—	26·4	
4·01	17 (?)	50·8	—	95	—	47·4	
3·66	—	3·3	—	73·5	—	92·0	
3·56	9·2	0	—	54·9	—	—	
3·46	0	0	—	66	—	—	
3·30	0	0	—	63·3	—	—	
3·10	0	0	—	68	—	—	
2·61	0	0	—	—	—	—	

Here R is given by the ratio

$$\frac{(\text{Total native HbCO} - \text{acid methaemoglobin}) \times 100}{\text{Total native HbCO}}.$$

The yield of "reversed" HbCO is plotted in Fig. 2, the amount of acid methaemoglobin in Fig. 1, curves 1 and 2.

If, on the other hand, the alkaline solution of protein is allowed to stand for some minutes and then neutralised with acid and filtered, a large amount is now found to be soluble. In other words the protein has "reversed" in the alkaline solution. When the protein solution is reduced with sodium hydrosulphite and treated as above, the haemoglobin is found to "reverse" more rapidly than when no reduction occurs. The following data illustrate the behaviour of methaemoglobin.

Into three 25 cc. graduated flasks were run 6·25 cc. McIlvaine buffer, p_H 2·9, and an equal volume of water, 0·5 cc. of protein solution, and 0·87 cc. of 0·1 *N* HCl. Each flask was allowed to stand for some time at the acid p_H, flasks 1 and 2 for 8 minutes, flask 3 for 25 minutes. (Time is a non-variant in such a system, and does not affect the final result.) At the end of the times concerned the protein solutions were neutralised with NaOH and the precipitate redissolved in the minimum amount of alkali and immediately neutralised with acid and filtered. The protein in the filtrate was then estimated as alkaline methaemoglobin. The solution in flask 3 was allowed to stand for 10 minutes in alkali before neutralisation with acid and filtration. Then the

dissolved material was estimated as with flasks 1 and 2. The results are set out below.

Flask No. ...	1	2	3
Time kept alkaline	0 mins.	0 mins.	10 mins.
Alkaline methaemoglobin	10 % (?)	0 % (?)	64 %

It is thus clear that for "reversion" to take place the protein must stand for some time in alkaline solution. At the end of this time soluble protein is obtained which shows a spectrum apparently identical with that of alkaline methaemoglobin. When the system is studied spectroscopically the spectrum of alkaline methaemoglobin is visible from the beginning. A solution of methaemoglobin treated exactly as before shows, on spectroscopic examination when in alkaline solution, the spectrum of alkaline methaemoglobin, even though it can be entirely precipitated on adding acid. This spectrum seems to exist initially at the same intensity as when the protein is entirely reversed. Thus the change in solubility occurring when the protein stands in alkali is not paralleled by any corresponding change in spectrum. If spectroscopic evidence be accepted as a criterion of denaturation, then native protein exists as alkaline methaemoglobin, even though this can be completely precipitated on neutralisation. Similarly the spectrum of reduced haemoglobin can be seen in such solutions and also that of carboxyhaemoglobin.

That no evident change in spectrum occurs on standing is shown by the following data.

In each of two 50 cc. graduated flasks were put 12·5 cc. of McIlvaine buffer p_H 2·21 and 2·47 respectively, 3·8 and 2·8 cc. of 0·1 *N* HCl and water to make the final volume 25 cc. Then 1 cc. of methaemoglobin solution was run in, and the protein allowed to stand for 30 minutes in acid solution. After this the solution was neutralised, the coagulum dissolved in the minimum alkali required, and the spectrum studied.

A series of 10 readings was then taken over a short range of time, the final reading being about 6 minutes after the first, and the composition of the protein mixture calculated from the mean of each adjacent two. The standards in the double-wedge trough were alkaline methaemoglobin in one side and an alkaline solution of parahaematin in the other. The results are tabulated below.

Reading No. ...	1	2	3	4	5
			p_H 2·21		
% alkaline methaemoglobin	64	62	66	66	67
			p_H 2·47		
% alkaline methaemoglobin	67	64	64	65	67

These values are, however, precisely those which would be obtained on "reversion" of the protein according to the method of Anson and Mirsky.

When the protein solution is reduced the spectrum of reduced haemoglobin can be seen, and, like alkaline methaemoglobin, reduced haemoglobin appears

to exist under those conditions in a solution which is entirely precipitated on bringing to the isoelectric point. The same holds for carboxyhaemoglobin.

When a study is made of the spectrum shown by haemoglobin solution brought originally to an acid p_H and then to p_H 7·8 to 8, it is found that the spectrum varies according to the p_H to which the protein was brought initially. Fig. 3 shows the relationship between the amount of alkaline methaemoglobin estimated spectroscopically and the initial p_H of the solution before making alkaline. The abscissae refer, not to the p_H of the protein solution during the time of estimation, but to the p_H of the McIlvaine buffer at which denaturation took place. As has been before mentioned the amount of native protein as estimated in this way seems independent of the solubility of the protein at the isoelectric point, *i.e.* of the length of time the protein has stood in alkali at p_H about 8. The details of these experiments are as follows. After having made the protein solution acid in precisely the manner described for the experiments with acid methaemoglobin and acid haematin, alkali was added in amount just sufficient to redissolve the precipitated protein. A clear solution was obtained which showed the spectrum of alkaline methaemoglobin. This was placed in the cell of the double-wedge trough and estimated against standards of alkaline methaemoglobin and an alkaline solution of parahaematin. Table II gives some numerical data obtained in this way.

Table II.

p_H	Alkaline methaemoglobin %	Carboxyhaemoglobin in alkaline solution %	Carboxyhaemoglobin in acid solution %
4·57	—	—	104
4·03	—	—	—
3·81	—	88·3	105
3·66	—	89·1	87·5
3·56	—	86·6	85·7
3·32	—	79·8	74
3·19	—	77·2	77·9
2·94	—	76·3	77·5
2·76	—	79	73
2·60	—	74·9	(?)
2·43	—	76·6	(?)
2·21	—	77·2	(?)
4·62	100	100	—
4·24	—	95	—
4·01	65·3	—	—
3·66	53·5	73·5	—
3·56	51·5	54·9	—
3·46	57·2	66	—
3·30	—	63·3	—
3·10	55·2	68	—
2·61	47·5	—	—
4·20	90·1	—	—
4·05	74	—	—
3·78	72	—	—
3·56	76	—	—
3·33	64·2	—	—
3·12	63·2	—	—
2·90	64	—	—

Similar curves have been obtained by bringing solutions of methaemoglobin to a series of definite p_H values in McIlvaine buffer and then "reversing" the solutions according to the method of Anson and Mirsky. This involves the treatment of the solution with alkaline cyanide reagent for a few hours, the p_H being about 8, followed by the separation of insoluble protein with half saturated ammonium sulphate, reduction of the filtered solution with sodium hydrosulphite and saturation with carbon monoxide. The reversed protein was estimated as carboxyhaemoglobin in the double-wedge trough, the standards being carboxyhaemoglobin and water. Table II shows the data obtained thus, and Fig. 3 illustrates the similarity between

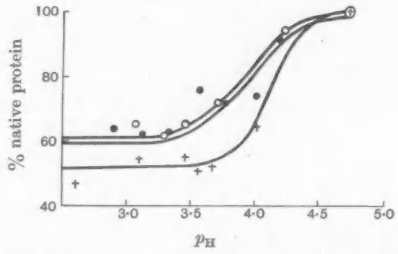

Fig. 3. A comparison of protein estimated as alkaline methaemoglobin after having been made acid in buffer, with that obtained as carboxyhaemoglobin after the cyanide treatment of Anson and Mirsky.

In order to obtain the solution of alkaline parahaematin the solution of haemoglobin was brought to a p_H of about 3 in phthalate buffer (Clark), and about 2·5 cc. *N* HCl added per 5 cc. phthalate buffer; the whole was warmed to about 60° and allowed to stand for about ½ hour, after which it was neutralised and made slightly alkaline. Points +, ● show the behaviour of protein so treated, while points ○ show the native material obtained after "reversion."

the amount of "reversed" protein and that estimated as alkaline methaemoglobin in the manner previously described. It is clear that the two curves coincide closely.

The problem may be attacked in a slightly different manner. When an acid solution of protein, such as has been studied above, is reduced with sodium hydrosulphite and aerated, the spectrum of oxyhaemoglobin can be seen. The bands rapidly disappear owing to the instability of the molecule at acid p_H. Partial neutralisation makes for greater stability, but it was never found possible to estimate the oxyhaemoglobin. When an acid solution of protein is reduced with sodium hydrosulphite in the presence of CO a solution is obtained which can be entirely precipitated on neutralisation. The solution shows a spectrum apparently identical with that of carboxyhaemoglobin, provided the p_H of the acid solution is not too low, when the spectrum of CO-reduced haematin appears; and on estimating the substance which resembles carboxyhaemoglobin against a standard of carboxyhaemoglobin in the double-wedge trough, a curve is obtained which coincides between p_H 4 and 5 with those obtained by reversion and as alkaline methaemoglobin. The spectrum of carbon monoxide-haemochromogen is very similar to that of

carboxyhaemoglobin and cannot be distinguished from it by the optical methods here used. Were, however, the protein entirely denatured and the substance estimated entirely CO-haemochromogen, it is hard to see how the amount of haemochromogen could vary (see Fig. 4) as no acid CO-reduced haematin could be observed at any p_H less than about 2·8. Before estimating the native protein the solutions were aerated thoroughly in each case in order to oxidise any haemochromogen present.

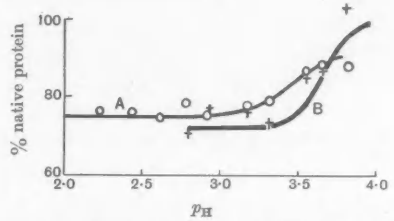

Fig. 4. Comparison of carboxyhaemoglobin obtained after treatment with alkaline cyanide with that estimated at acid p_H values down to p_H 2·75.

Points ○ refer to values obtained by making acid in McIlvaine buffer (12·5 cc. buffer in 50 cc. total volume), neutralising with $N/5$ NaOH and dissolving in about 5 cc. of cyanide solution, adding an equal volume of sat. $(NH_4)_2SO_4$ and filtering before converting to HbCO.

Points + were obtained by comparing the acid solution of protein with standards of carboxyhaemoglobin. The abscissae have different meanings for the two curves. For curve A the abscissae refer to the p_H to which the protein was brought in buffer, and for curve B to the p_H at which the estimation was carried out.

Table III gives data obtained in a series of preliminary experiments. The protein was about 5 % in concentration. To 1 cc. of this protein was added $N/5$ HCl in the amounts tabulated. Different volumes of $N/5$ NaOH were then added to this acid solution, reduction effected by adding $Na_2S_2O_4$, and then CO was passed in. The reduced solutions were then studied in the double-wedge trough, the standard solutions being carboxyhaemoglobin and water.

Table III.

cc. $N/5$ HCl added per 1 cc. protein	cc. $N/5$ NaOH added to acid mixture	% HbCO	cc. $N/5$ NaOH added	% HbCO
10	8·5	71·7	10	85
10	9·5	—	8·5	81
10	9·5	—	9·5	73·4
10	15·0	76·4	—	—
10	10·0	75·5	12·0	—
10	10·0	79·0	12·0	78·0
5	5·0	75·0	4·75	70·0
5	4·5	64·0	4·75	62·0

Where excess alkali was added the reduction was carried out by means of Stokes's reagent (alkaline reduction in 10 % sodium tartrate and dilute ferrous sulphate). The existence of HbCO at strongly acid p_H having been shown to be likely, the next series of experiments consisted in measuring the amount of HbCO obtainable between p_H 2·2 and 4·7. The curve thus obtained (Fig. 4) coincides closely with that obtained on reversion of the acid protein. Table II

compares the yield of native protein, obtained after reversion by the method of Anson and Mirsky, with the amounts estimated in the double-wedge trough at acid p_H as HbCO, and with the protein estimated as alkaline methaemoglobin.

Peculiarities in the behaviour of haemoglobin.

Chick and Martin found the temperature coefficient of the denaturation of haemoglobin to be 1·3 per 1°. Temperature, however, has little effect on the yield of native protein by reversion.

10 cc. $N/5$ HCl was added to 6·5 cc. 10 % oxyhaemoglobin. After 3 minutes the whole was neutralised with $N/5$ NaOH. The coagulum was washed with water and dissolved in KCN-buffer. The system was then reduced with $Na_2S_2O_4$ and CO bubbled through. Estimation of the amount of native protein showed that the effect of temperature is very small, the yield being about 43 % when the denaturation was carried out at 0°, and 32 % when at 40°.

The length of time the protein is kept at an acid p_H has no effect on the percentage recovery after the first minute when the temperature is 40°. This absence of effect of time is shown whatever the p_H; thus the amount of denatured protein does not increase on standing at, say, p_H 3·5 to the value obtained rapidly for p_H 2·8. There is no progressive increase on standing at acid reactions. The method used was as follows.

4 % ox methaemoglobin was acidified as in the previous experiment at a temperature of 40°. After definite intervals of time $N/5$ ammonia was run in. The precipitate was then dissolved in 2 % ammonia and reduced with Stokes's reagent. Aeration produced oxyhaemoglobin, which was estimated spectrophotometrically as in the case of other derivatives of haemoglobin. Fig. 5 illustrates the results obtained. Ox, sheep, and horse haemoglobins behaved

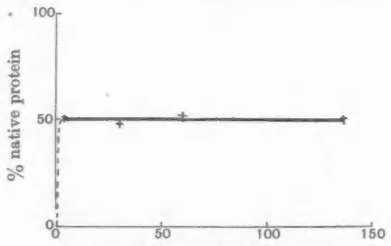

Fig. 5. Time-denaturation curve.

55 cc. 4 % ox methaemoglobin were acidified with 6·5 cc. $N/5$ HCl and $N/5$ ammonia was run in after various intervals.

alike in showing no progressive increase in denaturation on standing for various lengths of time at acid reaction at 0°, 20°, and 40°.

The influence exerted by foreign proteins seems to be slight. To 0·06 g.

methaemoglobin was added 0·06 g. crystalline egg-albumin; the control lacked the egg-albumin. The solutions were acidified with $N/5$ HCl to p_H 3. The undenatured protein was estimated as alkaline methaemoglobin in the spectrocolorimeter. The amounts were 70 % in the experiment and 68 % in the control. A similar result was obtained for laked blood.

Salts have but a small effect on the recovery of undenatured protein. To varying concentrations of NaCl in a constant volume of aqueous solution (14 cc.) 1 cc. of 5 % methaemoglobin solution was run in. 5 cc. of $N/5$ HCl were added to the solution of salt and methaemoglobin, and after 10 minutes 5 cc. $N/5$ NaOH. Then the protein was treated with alkaline cyanide, ammonium sulphate ($\frac{1}{2}$ sat.), and estimated as HbCO. The results of two experiments are shown in Fig. 6.

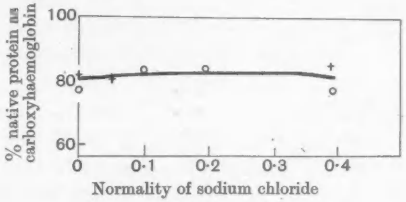

Fig. 6. Effect of increasing concentrations of NaCl on the yield of native protein.

The behaviour of the protein when subjected to repeated denaturation is of considerable interest. To protein in standard flasks was added HCl and after 5 minutes alkali in amount insufficient to neutralise the acid. For 5 cc. HCl were added 4·5 cc. $N/5$ NaOH. This was done once, twice, and three times. Then the protein was treated with cyanide, and estimated as HbCO. The yield of native material was never found to decrease on repetition of denaturation (Table IV).

Table IV.

No. of times acidified ...	1	2	3
	Percentage of native protein		
1	62·8	68	56
2	80	73	73
3	55	58	56

When the process is repeated but the protein made alkaline between each acidification, the yield of undenatured protein is still unaffected unless the denatured protein is separated from the native material, when the native residues denatured in the normal manner [Anson and Mirsky, 1929]. This observation has been confirmed by the author.

Discussion.

The experimental results described above present some remarkable features which it may be well to emphasise. The yields of "reversed" protein

seem largely independent of the temperature at which denaturation takes place; above a p_H of about 2·6 the extent of denaturation is completely independent of the p_H, and of the time of standing in acid solution. The steady state reached after a few minutes may be due to an equilibrium along the lines suggested by Anson and Mirsky, or to the whole of the protein being altered. It has been found that 100 % yield of native protein is never obtained, *i.e.* that some "irreversible" denaturation always takes place. (Wu and Lin state that they obtained complete "reversion" in one case.) It is very hard to conceive how the yield of "reversed" product can be independent of time and of repetition of acidification unless some alteration takes place in the whole of the protein. This change may be due to some denaturation in the classical sense of the word, followed by stabilisation of the remainder by the denatured product. If only part of the protein were denatured it might be expected that the residue of native material would become denatured as the protein stood in acid solution, and indeed the more so the longer it stood.

It is evident that there are two alternative views:

(*a*) the equilibrium view of Anson and Mirsky;

(*b*) the view that part of the protein is denatured and that this protects the remainder from further denaturation.

Now it has been shown that at acid p_H the spectrum of acid methaemoglobin can be detected, and, on reduction and aeration, that of oxyhaemoglobin. Some undenatured protein remains. Yet the amount of native material thus observed is always much smaller than that obtained by reversion. Some reversion may take place. But when the solution is made alkaline and the solution examined for alkaline methaemoglobin, the substance is seen in amount which accounts for the total native protein obtained, even though the whole protein is quantitatively precipitated on making isoelectric. Here we have direct evidence for the existence of some complex such as has been conceived in the second of the two suppositions. It may well be that in the acid solutions of protein studied the haematin group is entirely split off from the protein fraction, and that part of the free protein has become denatured, and has combined with the rest of the globin to form a complex stable to further treatment. On making alkaline it may be that the haematin fraction recombines with the globin and yields methaemoglobin, this being a rapid reaction. The alkali may further attack the complex between native and denatured protein, this being a slow reaction. Thus, if it be assumed that the complex between native and denatured material is insoluble at the isoelectric point, whether the protein is combined with haematin or not, we have a complete explanation of all the observed facts. For the alkaline methaemoglobin formed initially would thus be precipitated on neutralising at once; but after the slow dissociation had taken place between the native protein and the denatured, neutralisation would precipitate only the denatured fraction.

In the work here described the estimation of native protein falls ultimately

in every case on the formation of a conjugated protein. The validity of this procedure may be discussed with advantage. The question at issue is whether the formation of derivatives of globin, having spectra apparently identical with those of native protein, is a sufficient criterion for the existence of native globin. When a protein undergoes some secondary change, in every case it yields haemochromogen when coupled with reduced haematin [Anson and Mirsky, 1925]. Globin behaves as a typical protein in this respect. The action of acid, alkali, heat, light, shaking, urea, all give substances which have the haemochromogen spectrum. The least alteration in the protein appears to destroy its power of forming haemoglobin. This being the case it seems not improbable that were the protein concerned in these experiments altered in any fundamental way, the spectra of acid and alkaline methaemoglobin, of reduced and oxyhaemoglobin, and of carboxyhaemoglobin would not have been observed in solutions which could be entirely precipitated.

It has been suggested by Wu and Lin [1927] and by Anson and Mirsky [1929] that the sensitivity of oxyhaemoglobin to acids is greater than is that of reduced haemoglobin and they have suggested that the phenomena observed by Anson and Mirsky are due to the conjugation of the globin with the haematin. Anson and Mirsky have shown that similar effects may be obtained on acidifying solutions of globin, *i.e.* the prosthetic group may play some part in the phenomena, though not a decisive one. There is no evidence that like phenomena can be observed with other protein solutions.

Summary.

1. The formation of acid haematin from acid methaemoglobin has been studied as a function of p_H.

2. It has been found that some acid methaemoglobin appears to exist at p_H values at which the protein is entirely precipitable, and that hence reversion cannot account for all the native protein obtained after this procedure.

3. Curves have been obtained for the amount of soluble native protein obtained from methaemoglobin solutions brought to different p_H values.

4. On making an acid solution of protein neutral and then dissolving in the minimum of excess alkali, a solution is obtained which is entirely reprecipitated on neutralising with acid. If the solution be allowed to stand at an alkaline p_H the solubility increases, and finally a solution is obtained which is only partially precipitable on bringing to the isoelectric point.

5. A solution of protein made alkaline after having been acid shows the spectrum of alkaline methaemoglobin irrespective of whether the protein is soluble or insoluble at the isoelectric point, *i.e.* of the time through which the solution has stood alkaline. Estimation of the alkaline methaemoglobin in such solutions yields curves which coincide with those obtained after "reversion" by Anson and Mirsky's method.

6. Besides alkaline methaemoglobin, which precipitates entirely at the isoelectric point, solutions of reduced haemoglobin, oxyhaemoglobin, and

carboxyhaemoglobin can be made, which behave in a similar manner. The properties of all these solutions alter on standing, and the dissolved substances become only partially precipitable on neutralisation.

7. It is possible to obtain solutions at acid p_H which show spectra identical with that of carboxyhaemoglobin but which precipitate entirely on neutralisation. Estimation of the amount of native protein, measured by the intensity of this spectrum, yields curves similar to those obtained when the natural protein is estimated as alkaline methaemoglobin, or when it is subjected to the reversion procedure of Anson and Mirsky.

8. The methaemoglobin solution having been brought to a p_H of, say, 2·5, it has on no occasion been found possible to obtain all the material in the soluble native state, by any process whatever.

9. The author has confirmed the findings of Anson and Mirsky that the temperature at which denaturation takes place has little effect on the yield of native material after treatment with alkali, and that the extent of denaturation is unaffected by length of treatment with acid, after the first few minutes.

10. Denatured proteins combine with haematin to yield haemochromogen when the haematin is reduced, and parahaematin when it is oxidised. Yet the insoluble protein studied shows the spectrum of native derivatives of haemoglobin, reduced and oxy-haemoglobin, carboxyhaemoglobin, and alkaline methaemoglobin, in amount sufficient to account for all the native material obtained after treatment with alkaline cyanide. This phenomenon may be explained if it be assumed that there is formed a complex between the denatured fraction and the undenatured, this complex resembling denatured protein in being insoluble at the isoelectric point, and native protein in showing the typical spectrum of undenatured derivatives of haemoglobin.

I have much pleasure in expressing my thanks to Dr D. Keilin and Mr R. Hill for the help they have given me while this work was in progress, and to Sir F. G. Hopkins for the interest he has shown.

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CLXV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XX. ON THE PRODUCTION OF MANNITOL FROM HEXOSES AND PENTOSES BY A WHITE SPECIES OF *ASPERGILLUS*.

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The production of mannitol by bacterial fermentation has been extensively studied, since the organisms concerned are a source of trouble in the wine industry. Gayon and Dubourg [1901] showed that although glucose, sorbose, galactose, mannose, sucrose, maltose, lactose, raffinose and xylose are all fermented, laevulose alone yields mannitol. They found that arabinose and trehalose are not attacked. Peterson and Fred [1920, 1] reported that certain pentose-fermenting bacteria isolated from silage and manure also possess the power of producing mannitol. These organisms ferment glucose, galactose, mannose [Peterson and Fred, 1920, 2], xylose and arabinose [Fred, Peterson and Anderson, 1921], but again mannitol is obtained from laevulose alone, and not from any of the other sugars. With sucrose and raffinose some strains give traces of mannitol [Stiles, Peterson and Fred, 1925]. This fact was also observed by Gayon and Dubourg [1901], and it appears probable that fermentation of these sugars is not preceded by hydrolysis to monosaccharides, since invert sugar yields relatively large amounts of mannitol. It is most probable that mannitol arises by direct hydrogenation of one molecule of laevulose, coincidently with the oxidation of another molecule to lactic acid, acetic acid and carbon dioxide [Peterson and Fred, 1920, 1].

The presence of mannitol in the tissues of higher fungi was first discovered by Braconnot [1811], whose observation has been confirmed repeatedly and extended to the lower fungi. Obaton [1929], in a review on the evolution of mannitol in plants, studied in detail the production of mannitol and of trehalose in the mycelium of *Aspergillus niger*, but found no correlation between these two products, since factors affecting the one were without any effect on the other.

Recently Birkinshaw, Charles, Hetherington and Raistrick [1931] have shown that mannitol may be regarded as a product of mould fermentation. In the case of one white species of *Aspergillus* (Ac. 55), the yield of mannitol

approached 50 % of the glucose utilised. To obtain this result the rate of aeration must be carefully controlled. Since the source of mannitol is glucose, which on chemical reduction gives sorbitol, an intensive search was made for this alcohol, but no trace was discovered. The other metabolic products found in addition to mannitol were ethyl alcohol and carbon dioxide with traces of volatile and non-volatile acids.

In view of these results it was decided to extend this investigation, and the results of metabolism experiments on mannose, galactose, laevulose, xylose and arabinose are now reported. This work was restricted to one organism, *i.e.* that which gave the optimum yields of mannitol from glucose. In all experiments the medium consisted of 250 cc. of a 5 % solution of the sugar under investigation together with the usual Czapek-Dox salts, the medium being contained in a 750 cc. conical flask. It may be noted that this organism grows better in a medium where K_2HPO_4 is substituted for KH_2PO_4. The latter was used throughout, however, since the chance of a purely chemical decomposition of the sugar is thus decreased.

Experimental.

Carbohydrates.

A list of the sugars used in this work is given below, together with the observed optical rotations as a criterion of purity. The experiments with glucose, referred to in the introduction, were not repeated, but the results previously obtained [Birkinshaw, Charles, Hetherington and Raistrick, 1931] have been incorporated in Table II for comparison.

d-*Mannose* (British Drug Houses), as obtained gave $[\alpha]^{22}_{5461} + 18{\cdot}1°$ and $[\alpha]^{22}_{5790} + 16{\cdot}1°$ in water (c, 0·9948).

d-*Xylose* (Swann Chemical Co., Birmingham, Alabama), was recrystallised from aqueous alcohol. The final product gave $[\alpha]^{18}_{5461} + 22{\cdot}05°$ and $[\alpha]^{18}_{5790} + 19{\cdot}12°$ in water (c, 0·2900).

d-*Galactose* (Pfanstiehl), as obtained gave $[\alpha]^{19}_{5461} + 93{\cdot}5°$ and $[\alpha]^{19}_{5790} + 83{\cdot}0°$ in water (c, 1·000).

l-*Arabinose* (British Drug Houses), as obtained gave $[\alpha]^{20}_{5461} + 120{\cdot}4°$ and $[\alpha]^{20}_{5790} + 106{\cdot}9°$ in water (c, 1·1341).

d-*Laevulose* (Merck), as obtained gave $[\alpha]^{22}_{5461} - 107°$ and $[\alpha]^{22}_{5790} - 94{\cdot}7°$ in water (c, 2·009).

Medium.

Sugar	25·00 g.
$NaNO_3$	1·00 g.
KH_2PO_4	0·50 g.
KCl	0·25 g.
$MgSO_4.7H_2O$	0·25 g.
$FeSO_4.7H_2O$	0·005 g.
Water	to 500 cc.

The salts were dissolved separately and added to the solution of the sugar. 250 cc. of the medium were measured into each of two 750 cc. conical flasks and these were then sterilised by steaming for 30 minutes on each of three successive days.

Organism.

The organism used was an unnamed white species of *Aspergillus*, which was sent originally to Ardeer in 1923 by Dr Charles Thom, of the U.S. Department of Agriculture. It bore the Thom and Church Catalogue No. 4640·489, and the Ardeer Catalogue No. Ac. 55. Its carbon balance sheet was worked out [Birkinshaw, Charles, Raistrick and Stoyle, 1931] and it was then used for the preparation of mannitol from glucose [Birkinshaw, Charles, Hetherington and Raistrick, 1931]. All inoculations were made from beer-wort agar slopes grown at 25° for 5 days. A suspension in 5 cc. of distilled water of the heavily sporing mycelium from one test-tube culture was used for the inoculation of each flask of medium.

Incubation.

After inoculation, the two flasks were each fitted with a sterile rubber bung carrying an inlet glass tube which reached practically to the surface of the culture medium, and an outlet tube, the end of which only just passed through the bung. Each end of each tube was fitted with a cottonwool plug and the whole fitting—bung and glass tubes—was sterilised in the autoclave immediately before use. The inlet tube was connected to a controlled air supply and the outlet tube to a water seal, ensuring a closed system. The whole apparatus was maintained at a temperature of 25° (± 1°). Aeration was not commenced until a continuous felt of mycelium had been established (7–17 days). Thereafter 300 cc. of sterile air were passed into each flask daily, except Sundays. It had been shown previously that the omission of one day in seven only prolongs the period of incubation without affecting the ultimate result. After a few days of aeration, the thin film grew considerably and formed a thick white felt with a dry surface of sterile, woolly, aerial hyphae. This then remained unaltered, except for a slight wrinkling, until the supply of easily assimilable carbon became low, when the culture showed distinct signs of autolysis, shrinking and becoming water-logged. The flasks were then removed for examination.

With mannose, galactose and xylose the growth appeared normal and similar in character to that obtained on glucose. With arabinose, however, even after 3 months no sign of autolysis had appeared. One flask (*a*) was therefore removed after 93 days and examined. The other flask (*b*) was incubated for 11 more days and then, as autolysis became evident, it was also removed for examination. The residual sugar in both was high and, as will be seen later, the yield of mannitol was very much less than with glucose, mannose, galactose and xylose.

With laevulose the results were surprising, as after 3 weeks of incubation only rudimentary growth had occurred. A few tiny islets of mycelium formed very slowly, but even after 5 weeks these showed no signs of further development. It was thought possible that the laevulose might contain a trace of some toxic substance. That this was not so was shown by the abundant growth of other moulds on the same medium. Samples from one of the flasks were transferred to test-tubes, sterilised by one steaming for 30 minutes, and inoculated with six different species of *Aspergillus* and *Penicillium*. All of these showed normal growth in a few days.

A second attempt was made to grow the white *Aspergillus* on a fresh batch of the same medium. In spite of very heavy inoculations the same result was obtained—traces of growth which showed no appreciable development after the first fortnight. Since this organism prefers an alkaline medium, 2·0 cc. of sterile *N* NaOH were added to each flask after 3 weeks. This is equivalent to converting the acid phosphate into the dibasic salt. The flasks were shaken thoroughly to ensure mixing, but were not inoculated again since definite traces of growth were present. Ten days later a distinct improvement was apparent. In both flasks a ring of growth could be observed round the edge of the surface, together with a few discrete colonies. Aeration was now commenced, but the growth remained almost stationary, and 9 weeks later the flasks were removed for analysis.

Examination of metabolism solution.

The following procedure was adopted in all the experiments. The metabolism solution was filtered, without sterilisation, from the mycelium, which was washed 5 times with boiling water. Filtrate and combined washings from two flasks, corresponding to 500 cc. of original medium, were made up to 1 litre, constituting solution A. Aliquot portions of this were used for the various analyses and for the isolation of mannitol.

The collected results are given in Table II in which the results for glucose previously obtained [Birkinshaw, Charles, Hetherington and Raistrick, 1931] are incorporated.

The following estimations were carried out.

I. *Residual sugar*, (*a*) by polarimeter and (*b*) by Shaffer and Hartmann method.

(*a*) By polarimeter. The solution was polarised in a 4 dm. tube using both the mercury green and yellow lines, λ5461 and λ5790.

(*b*) By the Shaffer and Hartmann method. The normal procedure was followed without modification, the factors for the different sugars being determined by control estimations carried out on standard solutions of each sugar.

The results obtained by the two methods are given in item 4, Table II, and are in good agreement throughout.

II. *Acidity produced.* This was determined by titration with standard sodium hydroxide to phenolphthalein. The figures given in item 5, Table II,

Table I.

			Mannose	Galactose	Laevulose	Xylose	Arabinose (*a*)	Arabinose (*b*)
1	Concentration of sugar in standard metabolism solution A, calculated from mean polarimeter results (g. per 100 cc.)		Nil	0·516	1·46	0·290	0·735	0·750
2	Volume in cc. of metabolism solution A made up to 50 cc. with 3 g. of borax (solution B)		40·0	40·0	40·0	25·0	40·0	40·0
3	Concentration of sugar in solution B (g. per 100 cc)		Nil	0·413	1·168	0·145	0·588	0·600
4	Rotation of solution B in 4 dm. tube	λ 5461	+1·17°	+0·38°	−1·39°	+0·50°	+0·67°	+0·42°
		λ 5790		+0·36°	−1·27°	+0·45°	+0·57°	+0·39°
5	Rotation in 6·0 % borax of synthetic sugar solution of same concentration as in solution B	λ 5461	Nil	−0·59°	−1·39°	+0·04°	+0·29°	+0·29°
		λ 5790		−0·52°	−1·27°	+0·04°	+0·26°	+0·26°
6	Rotation due to mannitol (Item 4–Item 5)	λ 5461	+1·17°	+0·97°	Nil	+0·46°	+0·38°	+0·13°
		λ 5790		+0·88°	Nil	+0·41°	+0·31°	+0·13°
7	Percentage mannitol in solution B. Calculated from Item 6	λ 5461	0·730	0·60	Nil	0·27	0·22	0·084
		λ 5790		0·61	Nil	0·27	0·20	0·074
8	Total mannitol in metabolism solution A (g.)	λ 5461	9·13	7·5	Nil	5·4	1·4	0·53
		λ 5790		7·6	Nil	5·4	1·3	0·46

Table II.

		Glucose	Mannose	Galactose	Laevulose	Xylose	Arabinose (*a*)	Arabinose (*b*)
1	Incubation period (days)	77	65	72	94*	70	93	104
2	Aeration period (days)	70	49	55	62	60	83	96
3	Initial sugar (g.)	25·0	25·0	25·0	25·0	25·0	12·5	12·5
4	Residual sugar (g.) (*a*) by pol. λ 5461	—	Nil	5·13	14·6	2·90	3·66	3·75
	λ 5790	—	Nil	5·18	14·6	2·90	3·70	3·75
	(*b*) by Shaffer-Hartmann	0·34	Nil	4·55	14·6	—	3·57	4·15
					Decrease of		Decrease of	Decrease of
5	Acidity produced (cc. *N*/NaOH)	1·0	0·2	0·08	0·3	0·4	0·8	0·9
6	Mannitol estimated by polarimeter (g.)	8·4	9·1	7·5	Nil	5·4	1·35	0·50
7	Mannitol isolated (g.)	6·9	9·3	7·4	Nil	4·9	0·2†	0·1†
8	Percentage estimated yield of mannitol on sugar utilised	34·2	36·4	37·8	Nil	24·4	15·3	5·7
9	Percentage yield of mannitol isolated on sugar utilised	28·0	37·2	37·3	Nil	22·2	—	—

* NaOH (2 cc. *N*) added after 22 days' incubation. † Isolated as tribenzylidene compound

have been corrected for the original acidity of the medium. The acidity produced was practically negligible in all cases.

III. *Mannitol estimated by polarimeter.* The method used was that described by Raistrick and Young [1931] for the estimation of mannitol in fermentation solutions. This method is based on the fact that, although in aqueous solution mannitol gives a very small rotation, $[\alpha]^{20}_{5461} - 0{\cdot}49°$, in presence of 6·00 % borax a high positive rotation is given, the exact values of which vary with the concentration of mannitol and are given by the above authors. Since, however, the optical rotations of all the sugars used are greatly affected by the borax, a correction must be made for this effect. The procedure was as follows. The concentration of sugar in the metabolism solution A having been determined as described in Section I, a measured volume of the metabolism solution, together with 3·00 g. of borax ($Na_2B_4O_7 . 10H_2O$) was made up to a volume of 50 cc. and polarised. A solution of the sugar in question, in the same concentration as above, was also polarised in presence of 6·00 % borax. The difference between these two readings gave the rotation due to mannitol. The results obtained are given in Table I.

Isolation of mannitol.

A measured volume of the metabolism solution having been retained for the above estimations the remainder was used for the isolation of the product. 10 cc. of basic lead acetate solution were added and the precipitate was allowed to settle overnight. Excess lead was removed from the filtrate by H_2S and the solution was then concentrated *in vacuo* to 20–50 cc., the temperature of the water-bath being kept below 50°. At this stage a certain amount of crystalline material had usually separated. It was dissolved by warming and then 2 volumes of boiling absolute alcohol were added. On cooling, a voluminous crystalline mass of fine white needles was obtained. This was filtered, washed, dried and weighed. The mother-liquors were concentrated again and the process repeated until no further crystalline product separated.

The residual sugar remained in solution and did not interfere with the crystallisation of the mannitol, except in the case of arabinose. As shown in Table II, the two flasks of arabinose were examined separately, each solution being made up to a volume of 500 cc. No mannitol was isolated from either by direct crystallisation. With the first one a yield of 2·5 g. of white crystalline material was obtained, and estimations of reducing power and rotation showed that this product contained about 80 % of arabinose. The small amount of mannitol present could not be separated by direct means. It was, therefore, converted into the tribenzylidene derivative [Meunier, 1891] which was readily isolated and purified, as the sugar does not yield a benzylidene compound with HCl and benzaldehyde. The method, however, is not quantitative, only about 60 % of the calculated yield being obtained from synthetic mixtures of arabinose and mannitol. With the second arabinose metabolism solution no attempt was made to isolate a crystalline product directly. The concentrated

solution was desiccated *in vacuo* over P_2O_5 and then treated with HCl and benzaldehyde, by which means tribenzylidene mannitol was again obtained.

Each fraction of crystalline material obtained by the above treatment from the mannose, galactose and xylose metabolism solutions had M.P. between 158° and 163°. On recrystallisation from aqueous alcohol all fractions had M.P. 165–166° which was not depressed on mixing with an authentic specimen of pure mannitol (M.P. 166°). The optical rotation was also measured in each case and found to be $[\alpha]^{20}_{5461} - 0{\cdot}49°$ in water.

From each of the purified samples the tribenzylidene compound was prepared by the action of concentrated HCl and benzaldehyde [Meunier, 1891]. After recrystallisation from absolute alcohol the M.P. was 217–219°. The mixed M.P. with tribenzylidene mannitol was 217–219°. The optical rotation found was $[\alpha]^{17}_{5461} - 17{\cdot}3°$ and $[\alpha]^{17}_{5790} - 15{\cdot}6°$ in chloroform; Patterson and Todd [1929] give $[\alpha]^{25}_{5461} - 17{\cdot}5°$ and $[\alpha]^{25}_{5790} - 15{\cdot}5°$.

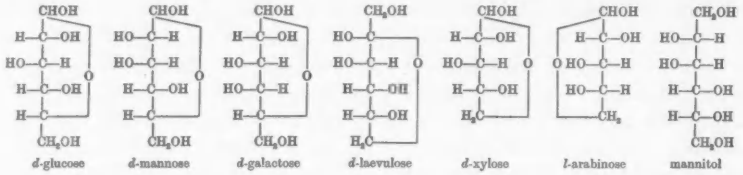
As a final confirmation of the identity of the mannitol a weighed amount of each specimen was polarised in 6·00 % borax solution when the calculated results agreed with the actual amounts used.

In the case of arabinose, as already stated, the mannitol was isolated as the tribenzylidene derivative. With laevulose no mannitol could be detected by the polarimetric method and none was isolated.

Discussion.

As is shown in Table II, the yield of mannitol from glucose, mannose and galactose is approximately the same, about 35 %. Xylose produced 24 %, but it is doubtful whether one can place any significance on this ratio, since Birkinshaw, Charles, Hetherington and Raistrick [1931] have shown that with unrestricted aeration the organism slowly utilises the mannitol formed. With arabinose the growth is less satisfactory and the yield of mannitol, although still appreciable, is poorer. Laevulose is apparently not a satisfactory source of carbon for this organism and is metabolised very slowly. The residual metabolism solution, however, contained only 60 % of the original sugar, so that 10 g. of laevulose had been utilised. In spite of this, no mannitol could be isolated and the rotation of the solution in 6·00 % borax confirmed the absence of even traces of this product (Table I). It may be that, since laevulose is so resistant to attack, any mannitol formed is broken down preferentially, but even were this the case, one would expect to find a small amount in the solution.

These results are rather unexpected, running as they do exactly contrary to the results of bacterial fermentation. In the latter case mannitol is produced from laevulose and not from any other hexose or pentose, while with this species of *Aspergillus* mannitol is obtained from all the sugars so far tested with the exception of laevulose. A consideration of the structural formulae renders the problem even more complex.



Mannose would naturally yield mannitol by direct hydrogenation, and for glucose one could postulate a Lobry de Bruyn type of transformation. Acceptance of such a hypothesis is rendered difficult by the laevulose results—since this sugar also gives mannose by the Lobry de Bruyn transformation, especially in alkaline solution—and becomes untenable when the pentoses are considered.

It might be claimed that the theory advanced to account for the alcoholic fermentation of sugar by yeast can be applied. Both the yeasts and the bacteria, however, show a fundamental difference in their metabolism as compared with the fungi. In the first cases the process is almost exclusively "catabolic" while with the fungi syntheses of an "anabolic" type are a striking characteristic. We have no evidence at present as to whether a hypothetical intermediate product, of the type of methylglyoxal, *etc.*, is transformed directly into mannitol. It appears at least equally possible that a hypothetical intermediate product is first built up into a reserve polysaccharide which is broken down as required and forms the immediate precursor of mannitol, which latter must then be regarded as a product of indirect synthesis. It is proposed to investigate this point by examination of the mycelium at various stages of growth, the mannitol produced being estimated at the same time.

Summary.

The metabolism of a species of white *Aspergillus* has been studied on synthetic media with controlled aeration. Mannitol is formed by this organism in considerable amounts (15–35 % of utilised sugar) from glucose, mannose, galactose, xylose and arabinose. The organism does not, however, produce mannitol from laevulose. These results are directly contrary to those reported for mannitol-producing bacteria.

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CLXVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXI. QUANTITATIVE EXAMINATION BY THE CARBON BALANCE SHEET METHOD OF THE TYPES OF PRODUCTS FORMED FROM GLUCOSE BY SPECIES OF BACTERIA.

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In an earlier paper of this series [Birkinshaw and Raistrick, 1931], a full description was given of a method for the determination of the products formed by micro-organisms by the carbon balance sheet method, with particular reference to moulds. A number of balance sheets in later papers [Raistrick *et al.*, 1931] show that by this means a useful general survey of the types of mould metabolism products can be obtained and may be used as a basis for the biochemical classification of these organisms. The method as originally evolved, however, was intended to cover the whole field of micro-organisms, and the present work was undertaken largely with a view to determining how far the original method would apply, and what modification would be necessary, in order to study the metabolic products of bacteria. In addition it was hoped to confirm and supplement in a quantitative manner the results obtained by Harden, Neuberg, Kluyver and other workers in this field. It was further anticipated that some of the organisms examined would show hitherto unsuspected amounts of some types of products, or of "carbon unaccounted for," and would thus open up interesting possibilities in the way of detailed investigation of the metabolism of the organism so selected.

Medium.

The Czapek-Dox-glucose medium used in the work on moulds was purposely chosen on account of its simplicity and its lack of any source of carbon other than glucose. Moulds are remarkably tolerant of acid, consequently the low p_H obtained with this medium when organic acids are produced does not markedly affect the growth or activity of these organisms. With bacteria, the

case is otherwise. They are known to be much more sensitive to acidity, and it was necessary, therefore, to adopt some method of neutralising the acid as formed. Both phosphate buffering and addition of calcium carbonate were tried, the latter proving more useful. Growth of *B. coli* (used as a test organism) on Czapek-Dox medium modified by addition of carbonate was unsatisfactory. It was considered that the form of nitrogen ($NaNO_3$) of the medium was probably unsuitable and a series of the commoner bacteria was therefore sown on to a Czapek-Dox medium containing the nitrogen in various forms, but in each case equivalent in nitrogen to the 0·2 % sodium nitrate of the original medium. Only 1 % of glucose was used so that an accumulation of acid was impossible, and calcium carbonate could thus be omitted without detriment to growth. The growth of the organisms after 5 days on the modified medium is indicated in Table I.

Table I. *Growth of stock strains of bacteria on modified Czapek-Dox solution containing various sources of nitrogen. Temp. 34°.*

		Time of incubation								
		5 days								48 hours
		Sod. nitrate	Sod. nitrite	Micro-cosmic salt	Amm. nitrate	Amm. sulphate	Amm. chloride	Calc. nitrate	Pot. nitrate	Micro-cosmic salt + $CaCO_3$
C 1.	*B. mesentericus*	+++	−	+++	−	+++	+++	−	−	+++
C 3.	*B. coli* (*Escherichia coli*)	−	−	+++	++	++	++	−	−	+++
C 4.	*B. proteus-vulgaris*	−	−	−	−	−	−	−	−	.
C 6.	*Bact. acidi-lactici* (*Escherichia acidi-lactici*)	−	−	+++	++	+	++	−	−	+++
C 12.	*Aerobacter cloacae*	−	−	+++	+	+++	+++	−	−	+++
C 13.	*B. figurans*	−	−	−	−	−	−	−	−	.
C 17.	*Serratia marcescens*	−	−	−	−	−	−	−		.
C 24.	*B. lactis-aerogenes* (*Aerobacter aerogenes*)	+++	−	+++	++	+++	.	−		++++
C 25.	Leather bac. Houston	++	−	+++	+	−	++	−	+++	++++
C 26.	Lactose acidifier (*Aerobacter*)	−	−	++	−	−	−	−	−	+
C 27.	Lactose fermenter (*Aerobacter*)	++	−	+++	+	+++	+++	−	+++	+++
C 28.	*B. mycoides*	−	−	+++	+++	+++	+++	−	+++	++
C 30.	Sporing rod bacillus	++	−	+++	+++	+	++	−	+++	++
C 33.	*Sarcina lutea*	−	−	−	−	−	−	−	−	.
C 34.	*B. coli* (*Escherichia coli*)	−	−	+++	+	+	++	−	−	+++

The medium contained only 1 % of glucose.

The microcosmic salt ($NaNH_4HPO_4.4H_2O$) medium showed definitely the best growth for the largest number of bacteria and was employed in a further trial. This time 1 % of calcium carbonate was added to the medium to determine whether this inhibited growth, since it would be necessary or at least desirable to use 5 % of glucose in the metabolism experiments with a correspondingly greater acid production. No inhibition was observed. The growth is shown in the last column of Table I.

It was thus evident that a number of organisms could be selected which grew rapidly on a medium containing merely inorganic salts apart from the glucose. A further batch of cultures shown in Table II yielded in most cases disappointing results, in respect of their growth on this medium.

Table II. *Growth of various stock strains of bacteria on modified Czapek-Dox solution containing various sources of nitrogen.*

Time of incubation 3 days at 34°.

	Sod. nitrate	Sod. nitrite	Amm. chloride	Amm. sulphate	Pot. nitrate	Calc. nitrate	Micro-cosmic salt	Amm. nitrate
C 38. *Streptococcus lacticus*	−	+	−	+	−	−	−	−
C 39. *B. guntheri* (*Streptococcus lactis*)	−	?	−	++	−	−	+	−
C 40. *B. bulgaricus* (*Lacto-bacillus bulgaricus*)	+	+	−	−	+	−	−	+
C 41. *B. acidophilus* (*Lacto-bacillus acidophilus*)	?	−	−	−	−	−	−	−
C 44. *B. pyocyaneus* (*Pseudomonas aeruginosa*)	++	−	++	+++	+++	−	+++	+
C 46. *Pseudomonas* (denitrifying)	+	−	−	−	−	−	+++	−
C 54. *B. acidi-lactici*	−	−	+	+	−	−	++	+
C 60. *B. faecalis-alcaligenes*	−	−	−	+	−	−	+	−
C 62. *B. acidi-lactici* (St Georges)	−	−	+	+	−	−	++	+
C 66. *B. butyricus* (Hueppe)	−	−	−	−	−	−	−	−
C 67. *B. mesentericus*	−	−	−	−	−	−	−	−
C 70. *B. asiaticus-mobilis*	++	−	+++	++	+	+	++++	+
C 73. *B. alkalescens*	−	−	+	−	.	−	++	−
C 80. *Mycobact. phlei*	−	−	−	−	.	−	−	−
C 81. Butter bac. (Rabinowitsch)	−	−	+	−	.	−	+	−
C 82. Moeller's "Mist" bacillus	−	−	++	+	.	−	++	+

At this stage it became evident that the number of species which would grow on a purely synthetic medium, containing only inorganic salts and glucose, was disappointingly small, and it appeared possible that the addition of amounts of organic nitrogen which would introduce carbon in quantities representing only a small percentage of that present as glucose would render a much larger number of bacteria available, whilst not appreciably affecting the carbon balance. Large amounts of organic nitrogen would be out of the question, since they would vitiate the carbon balance or, at least, necessitate a cumbersome technique. Growth on media obtained by addition of 2 drops of 10 % peptone, 10 % asparagine, caseinogen digest or double strength yeast water to 10 cc. of the Czapek-Dox microcosmic salt medium was compared for a series of organisms, which failed to grow in the absence of organic nitrogen (see Table III).

Table III. *Effect of additions of small amounts of organic nitrogen to modified Czapek-Dox medium.*

Incubated 4 days at 34°.

	Peptone	Asparagine	Caseinogen digest	Yeast water
C 38. *Streptococcus lacticus*	+	−	+	−
C 39. *B. guntheri* (*Streptococcus lactis*)	++	−	−	+
C 40. *B. bulgaricus* (*Lacto-bacillus bulgaricus*)	+	−	−	−
C 41. *B. acidophilus* (*Lacto-bacillus acidophilus*)	−	−	−	−
C 60. *B. faecalis-alcaligenes* (*Alcaligenes faecalis*)	+	−	++	−
C 66. *B. butyricus* (Hueppe)	++	−	++	−
C 67. *B. mesentericus*, No. 4	+	−	+++	++
C 80. *M. phlei*	+	−	+	+++
C 81. Butter bac.	++	++	++	++

As the addition of peptone appeared to give the best results it was adopted as a standard constituent to be added to the medium. The amount was fixed at 0·2 % corresponding to only 4 % by weight of the sugar, since this produced a considerable increase in growth whilst not interfering appreciably with the carbon balance sheets.

With the series of bacteria growing best at 25–30° the inorganic nitrogen proved relatively inefficient though here again the microcosmic salt was most favourable. The addition of peptone again rendered the medium much more suitable for bacterial growth.

The Czapek-Dox medium had been rather arbitrarily selected as the synthetic medium owing to its previous use for mould metabolism experiments. In order to justify its retention it was compared with other synthetic media, notably those of Prazmovski and Raulin (the latter *minus* tartrate). No advantage was obtained by the use of these two media, in fact the Czapek-Dox microcosmic salt and peptone medium was definitely superior.

The modified Czapek-Dox media employed were then as follows.

Medium A.

Microcosmic salt ($NaNH_4HPO_4$, $4H_2O$)	4·921 g.
K_2HPO_4	1·00 g.
KCl	0·50 g.
$MgSO_4$, $7H_2O$	0·50 g.
$FeSO_4$, $7H_2O$	0·01 g.
Glucose	50·00 g.
$CaCO_3$	28·00 g.
Distilled water	1000 cc.

250 cc. of medium, in a 750 cc. conical flask, were used in each experiment.

Medium B. To the above medium was added 0·2 % of peptone for certain species in order to increase the range of species which could be employed.

Medium C. This medium represented an attempt to eliminate the use of calcium carbonate by the employment of a phosphate solution of high buffering power. Medium C contains 8·70 g. of dipotassium phosphate per litre in place of 1 g. as in Medium A, the calcium carbonate being omitted. Thus glucose represents the only source of carbon, resulting in simplification of the balance sheet. Only two examples of its use are given.

Modifications in technique.

Certain departures from the standard technique as evolved for the study of moulds were necessitated by the different habits of growth of bacteria and by the difference in the medium used.

When calcium carbonate was employed, a known amount was sterilised dry in a plugged tube which was weighed, and the contents were added to the metabolism flask under aseptic conditions. The empty tube and plug were next weighed and the exact weight of carbonate obtained by difference. The flask was then sown with the organism to be examined and incubated in an apparatus similar to that used in the mould work.

In order to allow for a considerable evolution of carbon dioxide from the action of organic acids on the calcium carbonate present, a new type of potassium hydroxide absorption bulb was fitted immediately after the first

sulphuric acid bubbler. In this the carbon dioxide entering was allowed to impinge on the surface of the potassium hydroxide solution, the delivery tube being cut off about half an inch above the surface of the absorbent liquid. Much of the carbon dioxide was removed in this manner without any danger of blocking the tube with solid potassium bicarbonate, as occasionally happened with the moulds.

The air supplied to aerobic bacteria was the same as for moulds, *viz.* about 500 cc. daily. The air inlet tube in most cases dipped below the surface of the medium, so that the air on entering bubbled through the liquid and to some extent agitated the calcium carbonate. If the bacteria under examination were known to be film formers, however, the standard mould procedure was adopted, the air being admitted above the liquid surface. In the case of anaerobic bacteria the air was swept out of the apparatus with a current of nitrogen, and no further attention was given until the end of the experiment when the gaseous metabolism products were again removed by nitrogen.

After the incubation had proceeded for the length of time necessary to metabolise the greater part of the sugar as determined from a control experiment the apparatus was swept out with a current of air (or nitrogen) and then the several portions of the apparatus treated substantially as in the mould experiments. Only in the case of the flask contents was a modification necessary.

No preliminary titration of the flask contents was undertaken owing to the presence of calcium carbonate. An idea of the amount of acid formed is obtained from the amount of carbonate which disappears during the experiment. This approximate figure is inserted in the balance sheet in place of the figure obtained from the titration of the metabolism solution.

The contents of the flask are filtered through a filter-paper on which is distributed a weighed amount (about 0·5 g.) of "Supercel Hyflo" in place of kieselguhr. This was found to give easier filtration and to be practically carbon-free. A further weighed amount (1·5 g.) was added to the metabolism solution which was well shaken before filtration. The dried bacterial or other insoluble residue, together with the unused calcium carbonate and the supercel, remains on the paper. After drying it peels off readily and is weighed and ground up in a mortar. The residual carbonate present is determined by warming a portion of the mixture with dilute acid in the wet combustion apparatus, the carbon dioxide being determined in the usual way. On another portion a total carbon estimation is performed. In this way the value for "carbon in bacteria" is obtained.

The main filtered solution is made up to a known volume (500 cc.) and the determinations necessary to construct a carbon balance sheet are made.

It was found that lactic acid and butylene glycol, which would normally be included in the "carbon unaccounted for" are such frequent products of bacterial metabolism that the separate determination of them was necessary, in order to decide whether other products were present in this fraction.

Procedure.

The residue from the determination of the "volatile neutral carbon" (for which 100 cc. is taken in the ordinary method) is made up to 100 cc. and 25 cc. removed. This neutral solution is extracted with ether for some 12 hours in a continuous apparatus. This has the effect of removing all the butylene glycol (along with any compounds of similar nature, if present). The extract is set aside for glycol determination and the residue is acidified with phosphoric acid and the extraction continued for another 12 hours. The resulting extract is used for the estimation of lactic acid.

Determination of butylene glycol.

Preliminary tests of the method suggested by Malaprade [1928] for polyhydric alcohols showed that butylene glycol when oxidised by periodic acid behaves in a similar manner to ethylene glycol. One molecule of butylene glycol requires 1 atom of periodate oxygen according to the equation:

$$CH_3.CHOH.CHOH.CH_3 + HIO_4 = 2CH_3CHO + HIO_3 + H_2O.$$

Although this method would not in itself distinguish between butylene glycol and other polyhydric alcohols, it is a valuable aid to the construction of the carbon balance sheets when applied with discrimination and supplemented in doubtful cases by specific tests for, or by the isolation of, butylene glycol.

A standard solution of potassium periodate was made up such that on acidification and addition of excess potassium iodide it yielded a $N/10$ solution of iodine (calculated on the original volume). A known amount of the material extracted by ether from the neutral metabolism solution containing not more than 0·05 g. of butylene glycol was treated with 100 cc. of the periodate solution and 10 cc. of 10 % sulphuric acid. The mixture was allowed to stand overnight and then an excess of potassium iodide added and the free iodine titrated with $N/10$ thiosulphate. This titration figure must be more than 75 cc. which would correspond with the total conversion of the reagent.

Determination of lactic acid.

The method involving distillation with dilute permanganate and titration of the acetaldehyde evolved has been frequently modified, though the original form of the method is associated with the names of von Fürth and Charnass [1910].

The actual procedure is as follows.

A known amount of the solution containing 0·5–5·0 mg. of lactic acid is introduced into a Claisen flask together with 10 cc. of 10 % sulphuric acid. The flask is attached to an efficient Liebig condenser and a receiver consisting of a Volhard flask containing 20 cc. of $N/20$ sodium bisulphite. The Claisen flask is heated in a boiling water-bath and a current of steam passed in through

one neck. Through the other neck $N/200$ potassium permanganate solution is slowly dropped in until the liquid in the flask attains a permanent pink colour.

After a further 5 minutes the steam is shut off, the condenser washed down into the bisulphite and the contents of the Volhard flask washed out into a conical flask. Starch is added and the excess bisulphite is titrated with $N/20$ and finally $N/1000$ iodine until a pale blue colour is obtained. Excess of saturated sodium bicarbonate solution is then added to decompose the aldehyde-bisulphite compound and the solution is titrated with $N/1000$ iodine to re-establish the colour. With 1 cc. of 0·0997 % lithium lactate duplicate titrations obtained were 20·2 and 20·9 cc. of $N/1000$ iodine. The mean 20·55 cc. corresponds to 0·986 mg. lithium lactate, giving an error of 1 %.

The one difficulty which remains is due to the slight volatility of lactic acid. During the distillation of the volatile acids a certain amount of the lactic acid present distils over and is recorded as volatile acids. Since a separate lactic acid estimation is performed on another portion of the metabolism solution the carbon accounted for is slightly higher than it should be. This is borne out by the fact that in some cases the total carbon accounted for is higher than that present in solution. The error introduced in this manner is however fairly small. In distillation tests carried out with lactic acid present in about double the amount that was present in any metabolism experiment, lactic acid distilling over under the conditions of the experiment corresponded to 0·4 g. of carbon. This would, of course, represent a maximum error; it will be seen from the balance sheets that the actual error is usually considerably less, since the total volatile acid is frequently less than this figure even when lactic acid is present.

An attempt was made to calculate the respiration coefficient as for the moulds but when calcium carbonate is present there is difficulty in distinguishing between carbon dioxide arising from the carbonate and that due to respiration. It was assumed, however, for this purpose that all the carbonate disappearing had given rise to the theoretical amount of carbon dioxide, which was therefore deducted from the total carbon dioxide recovered.

Since the weight of bacteria was only determinable indirectly and was in any case small, this weight could not be accurately obtained. Hence the figure for the % carbon in bacteria was unreliable and was not determined although, of course, the total carbon in the bacteria was estimated.

It was found that a number of the organisms which grow quite well on the medium selected are unable to metabolise more than a small portion of the sugar supplied. Consequently, the standard procedure adopted was to carry out a control experiment with each organism giving good growth on the synthetic medium.

In these control experiments (Table IV) the organism was sown into a flask, fitted with tubes for aeration, and containing the medium as used in the metabolism experiments. It was incubated at a suitable temperature,

Table IV.

Cat. No.	Name	Source	Medium	Temp. °C.	Days	Sugar % Start	Sugar % Finish
C 39.	*B. güntheri* (*Streptococcus lactis*)	N.C.T.C. No. 661	B	34	26	5·02	3·16
C 38.	*Streptococcus lacticus*	N.C.T.C. No. 1551	B	34	21	5·17	3·74
C 87.	*Leuconostoc* species	Isolated from molasses at Ardeer	B	34	16	5·02	1·83
C 79.	*M. flavus* (Flugge)	N.C.T.C. No. 1629	B	28	14	5·39	5·25
C 78.	*M. roseus* (Bumm)	N.C.T.C. No. 195	B	28	28	4·91	4·63
C 44.	*B. pyocyaneus* (*Pseudomonas aeruginosa*)	N.C.T.C. No. 1999	A	34	8	5·14	4·68
C 44.	*B. pyocyaneus* (*Pseudomonas aeruginosa*)	N.C.T.C. No. 1999	B	34	31	5·02	2·44
C 46.	*Pseudomonas* (dentrifying)	N.C.T.C. No. 933	A	34	6	5·14	4·56
C 35.	*B. rossicus* Kell. (*Cellulomonas rossica*)	N.C.T.C. No. 1655	A	34	11	5·14	4·83
C 4.	*B. proteus-vulgaris* (*Proteus vulgaris*)	N.C.T.C. No. 67	B	34	6	5·21	3·66
C 47.	*B. phytophthorus* Appel	N.C.T.C. No. 1985. Dr E. F. Smith	B	28	20	5·50	3·12
C 45.	*B. aroideae* Townsend (*Erwinia aroidea*)	N.C.T.C. No. 1987. Dr E. F. Smith	B	28	31	5·35	0·10
C 51.	*B. carotovorus* Jones (*Erwinia carotovora*)	N.C.T.C. No. 1975. Dr E. F. Smith	B	28	27	4·56	1·90
C 43.	*B. marginale* N. A. Brown (*Phytomonas marginale*)	N.C.T.C. No. 1981. Dr E. F. Smith	B	28	15	5·12	4·70
C 50.	*B. campestre* Pammel (*Phytomonas campestre*)	N.C.T.C. No. 1958. Dr F. E. Smith	B	28	17	5·45	4·60
C 63.	*B. hyacinthi* Wakker	N.C.T.C. No. 387	B	28	26	5·54	4·68
C 64.	*B. aptatum* Brown and Jamieson (*Phytomonas aptata*)	N.C.T.C. No. 1977. Dr E. F. Smith	B	28	28	4·89	2·41
C 65.	*B. malvacearum* E. F. Smith (*Phytomonas malvacera*)	N.C.T.C. No. 1960. Dr E. F. Smith	B	28	11	5·31	5·20
C 75.	*B. cannae* Bryan (*Phytomonas cannae*)	N.C.T.C. No. 1956. Dr E. F. Smith	B	28	15	5·49	5·48
C 54.	*B. acidi-lactici*	N.C.T.C. No. 87	B	34	6	5·02	2·24
C 6.	*B. azidi-lactici* Grotenfelt, Hueppe (*Escherichia acidi-lactici*)	N.C.T.C. No. 413	A	34	6*	4·91	2·19
C 62.	*B. acidi-lactici* (St Georges)	N.C.T.C. No. 123	B	34	10	5·02	0·36
C 34.	*B. coli* Esch. (*Escherichia coli*)	N.C.T.C. No. 86	A	34	6*	4·91	2·51
C 37.	*Esch.* species	Isolated from milk at Ardeer	A	34	6*	4·91	1·09
C 26.	*Esch.* species	Isolated from milk at Ardeer	A	34	6*	4·91	3·12
C 24.	*B. lactis-aerogenes* Esch. (*Aerobacter aerogenes*)	N.C.T.C. No. 418	A	34	9*	4·91	0·02
C 24.	*B. lactis-aerogenes* Esch. (*Aerobacter aerogenes*)	N.C.T.C. No. 418	C	34	7*	4·93	1·37
C 12.	*Aerobacter cloacae*	Isolated at Ardeer	A	34	6*	4·91	0·86
C 12.	*Aerobacter cloacae*	Isolated at Ardeer	C	34	6*	4·93	2·77
C 25.	Leather bacillus (Houston)	N.C.T.C. No. 672	A	34	6*	4·91	0·08
C 27.	*Aerobacter* species	Isolated at Ardeer	A	34	6*	4·91	0·08
C 70.	*B. asiaticus-mobilis* Castellani	N.C.T.C. No. 1747	A	34	2	5·14	0·0
C 73.	*B. alkalescens* Andrewes	N.C.T.C. No. 1601	B	34	17	5·02	1·95
C 60.	*B. fecalis-alcaligenes* Petruschky (*Alcaligenes fecalis*)	N.C.T.C. No. 415	B	34	8	5·02	4·64
C 72.	*B. ureae* Leube	N.C.T.C. No. 1654	B	28	26	5·06	2·13
C 98.	*B. albolactis* (Löffler) Migula (298)	American Type Culture Collection, No. 3	B	28	17	5·22	2·29
C 67.	*B. mesentericus* No. 4	N.C.T.C. No. 2589	B	34	17	5·02	3·35
C 1.	*B. mesentericus*	Isolated at Ardeer	B	34	8	5·02	4·31
C 90.	*B. megatherium* De Bary	N.C.T.C. No. 654	B	34	10	4·77	3·92
C 30.	Rod bacillus, oval spores	Isolated at Ardeer	B	34	21	5·17	3·39
C 28.	*B. mycoides*	Isolated at Ardeer	B	34	21	5·17	2·86
C 69.	*B. bifermentans* Tissier and Martelly (*Clostridium bifermentans*)	N.C.T.C. No. 1340	B	34	15	4·77	3·38
C 96.	*Clostr. saccharobutyricum* Beijerinck	American Type Culture Collection, No. 860	B	34	7	5·17	1·47
C 94.	*Clostr. pasteurianum* Winogradsky	American Type Culture Collection, No. 861	B	34	7	5·17	1·39
C 80.	*Myc. phlei* Söhn.	N.C.T.C. No. 1471	B	34	21	5·17	4·23
C 82.	Moeller's "Mist" Bacillus (*Myc. stercusis*)	N.C.T.C. No. 526	B	34	17	5·02	3·97
C 81.	Bacillus (acid fast) from butter (butter bacillus of Rabinowitsch)	N.C.T.C. No. 524	B	34	8	5·02	4·64

N.C.T.C. refers to the British National Collection of Type Cultures, Lister Institute.

samples being withdrawn aseptically from time to time for sugar determination. Only if the sugar disappeared at a reasonable rate was it considered worth while to perform a complete metabolism experiment. In the event of the metabolism experiment being carried out, the control gave a useful indication as to the rate of progress of the fermentation, so that the correct time could be allotted for the incubation period.

Preliminary controls are lacking for some of the organisms used for the earlier balance sheets. In these cases the balance sheet figures are inserted in the table for the sake of completeness. Where this is done it is indicated by an asterisk. The results of the carbon balance sheet determinations are summarised in the Tables V–IX.

Table V.

	Leuconostoc species	*B. aroideae*	*B. carotovorus*	*B. proteus-vulgaris*	*B. proteus-vulgaris*
Medium used	B	B	B	B	B
Catalogue number	C 87	C 45	C 51	C 4	C 4
Temperature	34°	28°	28°	34°	34°
Incubation period in days ...	13	26	26	6	27
Carbon balance sheet (g.)					
Carbon in solution (start)	5·109	5·189	5·189	5·214	5·189
„ added as $CaCO_3$	0·840	0·837	0·839	0·838	0·835
Total carbon (start)	5·949	6·026	6·028	6·052	6·024
Carbon in H_2SO_4	0·002	0·007	0·003	0·002	0·007
„ in CO_2	0·651	1·324	0·576	0·383	0·435
„ in bacteria	0·100	0·233	0·059	0·081	0·167
„ in residual $CaCO_3$	0·450	0·579	0·450	0·625	0·527
„ in solution	4·679	3·786	4·848	4·941	4·840
„ accounted for	5·882	5·929	5·936	6·032	5·976
„ accounted for (%)	98·9	98·4	98·5	99·7	99·2
Analysis of solution (g.)					
Carbon in residual glucose	2·266	0·042	2·416	3·662	3·328
„ in CO_2 in solution	0·006	0·006	0·005	0·006	0·005
„ in volatile acids	0·457	0·288	0·475	0·344	0·313
„ in non-volatile acids (Ca ppt.)	0·146	0·336	0·323	0·084	0·117
„ in volatile neutral compounds	0·194	0·269	0·225	0·237	0·420
„ in synthetic compounds ...	0·063	0·119	0·101	0·057	0·053
„ as butylene glycol	0·311	2·136	Nil	—	0·168
„ as lactic acid	1·008	0·288	1·152	—	0·360
Total carbon accounted for	4·451	3·484	4·697	4·390	4·764
Total carbon in solution	4·679	3·786	4·848	4·941	4·840
Carbon unaccounted for (by difference)	0·228	0·302	0·151	0·550	0·076
Residual glucose (%)					
Glucose (by polarimeter)	0·828	Nil	1·187	1·774	1·653
„ (by Shaffer-Hartmann) ...	1·133	0·021	1·208	1·831	1·664
„ (by Wood-Ost)	1·104	0·021	1·182	1·805	1·692
Acids					
Titration (calculated) (cc. $N/1$) ...	65·0	43·0	64·8	35·5	51·3
Volatile acids (cc. $N/1$)	12·19	6·75	15·98	14·4	13·4
Barium salts (weight in g.)	2·050	1·083	2·584	1·996	1·692
Calcium salts (weight in g.)	0·451	1·020	0·918	0·104	0·098
Volume of oxygen absorbed (cc.) ...	395	590	350	93	210
Respiration coefficient	1·23	3·37	10·0	3·41	1·13
Volume of hydrogen evolved (cc.) ...	Nil	Nil	Nil	Trace	Nil

The bacterial strains tested in the control experiments (Table IV), and those employed in the complete metabolism experiments (Tables V to IX)

Table VI.

	B. acidi-lactici	*B. acidi-lactici* Groten-felt Hueppe	*B. acidi-lactici* (St Georges)	*B. coli* (*E. coli*)	*Escherichia* species from milk	*Escherichia* species from milk
Medium used	B	A	B	A	A	A
Catalogue number	C 54	C 6	C 62	C 34	C 37	C 26
Temperature	34°	34°	34°	34°	34°	34°
Incubation period in days ...	6	6	6	6	6	6
Carbon balance sheet (g.)						
Carbon in solution (start)	5·214	4·912	5·214	4·912	4·912	4·912
,, added as $CaCO_3$...	0·840	0·833	0·837	0·839	0·835	0·834
Total carbon (start)	6·054	5·745	6·051	5·751	5·747	5·746
Carbon in H_2SO_4	0·005	0·003	0·003	0·004	0·004	0·002
,, in CO_2	0·920	0·566	0·791	0·590	0·838	0·411
,, in bacteria	0·202	0·091	0·205	0·262	0·156	0·054
,, in residual $CaCO_3$	0·200	0·388	0·331	0·288	0·207	0·549
,, in solution	4·568	4·593	4·636	4·617	4·443	4·653
,, accounted for	5·895	5·641	5·966	5·761	5·648	5·669
,, accounted for (%)	97·4	98·2	98·6	100·2	98·3	98·6
Analysis of solution (g.)						
Carbon in residual glucose	1·101	2·190	2·030	2·508	1·092	3·121
,, in CO_2 in solution	0·008	0·006	0·003	0·004	0·005	0·004
,, in volatile acids...	0·524	0·253	0·719	0·434	0·659	0·381
,, in non-volatile acids (Ca ppt.)	0·311	0·263	0·097	0·105	0·115	0·045
,, in volatile neutral compounds	0·539	0·269	0·436	0·287	0·376	0·186
,, in synthetic compounds ...	0·074	0·052	0·032	0·040	0·047	0·038
,, as butylene glycol	Nil	Nil	Nil	0·022	0·042	0·072
,, as lactic acid	1·395	1·260	0·990	0·882	1·518	0·900
Total carbon accounted for	3·952	4·293	4·307	4·282	3·854	4·747
Total carbon in solution	4·568	4·593	4·636	4·617	4·443	4·653
Carbon unaccounted for (by difference)	0·616	0·300	0·329	0·335	0·589	—
Residual glucose (%)						
Glucose (by polarimeter)	0·600	1·280	0·988	1·345	0·662	1·539
,, (by Shaffer-Hartmann) ...	0·551	1·095	1·015	1·254	0·546	1·560
,, (by Wood-Ost)	0·585	1·128	1·019	1·290	0·598	1·525
Acids						
Titration (calculated) (cc. $N/1$) ...	106·7	74·2	84·4	91·9	104·7	47·5
Volatile acids (cc. $N/1$)	20·2	7·50	26·1	16·6	23·5	14·9
Barium salts (weight in g.)	2·882	1·331	4·019	2·400	3·457	2·182
Calcium salts (weight in g.)	0·812	0·772	0·055	0·034	0·146	—
Volume of oxygen absorbed (cc.) ...	119	13	131	125	66	77
Respiration coefficient	4·40	17·3	4·06	0·57	6·05	3·07
Volume of hydrogen evolved (cc.) ...	285	82·5	388	Nil	165	Nil

are listed by the names under which they were received, the source from which they were obtained being indicated in each case. No attempt has been made to study these strains from the systematic point of view; nor, in the present confused state of bacterial nomenclature, has it seemed desirable to insert alternative names, in accordance with any one of the systems in current use.

The strains brought together in Tables VI and VII are certainly closely related. Those in Tables V and IX include widely separated species. Our

Table VII.

	B. lactis-aero-genes	*B. lactis-aero-genes*	*A. cloacae*	*A. cloacae*	Leather bacillus Houston	*Aero-bacter* species from milk
Medium used	A	C	A	C	A	A
Catalogue number	C 24	C 24	C 12	C 12	C 25	C 27
Temperature	34°	34°	34°	34°	34°	34°
Incubation period in days ...	9	7	6	6	6	6
Carbon balance sheet (g.)						
Carbon in solution (start)	4·912	4·928	4·912	4·928	4·912	4·912
„ added as $CaCO_3$	0·835	—	0·831	—	0·836	0·839
Total carbon (start)	5·747	4·928	5·743	4·928	5·748	5·751
Carbon in H_2SO_4	0·019	0·004	0·010	0·005	0·004	0·006
„ in CO_2	1·677	0·864	1·131	0·478	1·251	1·254
„ in bacteria	0·256	0·090	0·183	0·003	0·168	0·138
„ in residual $CaCO_3$	0·693	—	0·504	—	0·306	0·371
„ in solution	2·750	3·784	3·842	4·494	3·948	3·885
„ accounted for	5·395	4·742	5·670	4·980	5·677	5·654
„ accounted for (%)	93·9	96·2	98·7	101·0	98·8	98·3
Analysis of solution (g.)						
Carbon in residual glucose	0·017	1·366	0·863	2·768	0·081	0·083
„ in CO_2 in solution	0·008	0·006	0·006	0·006	0·006	0·006
„ in volatile acids...	0·155	0·106	0·326	0·198	0·648	0·618
„ in non-volatile acids (Ca ppt.)	0·062	0·341	0·104	0·483	0·072	0·030
„ in volatile neutral compounds	0·687	0·518	0·684	0·362	0·604	0·527
„ in synthetic compounds ...	0·020	0·008	0·016	0·003	0·057	0·048
„ as butylene glycol	1·527	0·912	1·603	0·465	0·661	0·968
„ as lactic acid	0·157	0·504	0·128	0·072	1·710	1·710
Total carbon accounted for	2·633	3·761	3·730	4·357	3·839	3·990
Total carbon in solution	2·750	3·784	3·842	4·494	3·948	3·885
Carbon unaccounted for (by difference)	0·117	0·023	0·112	0·137	0·109	—
Residual glucose (%)						
Glucose (by polarimeter)	0·113	0·773	0·462	1·418	0·275	0·243
„ (by Shaffer-Hartmann) ...	0·008	0·683	0·432	1·384	0·203	0·042
„ (by Wood-Ost)	—	0·686	0·435	1·378	0·192	0·038
Acids						
Titration (calculated) (cc. $N/1$) ...	23·6	15·0	54·6	8·0	88·3	77·9
Volatile acids (cc. $N/1$)	—	3·37	20·3	6·88	18·4	12·8
Barium salts (weight in g.)	0·085	0·450	2·579	1·195	2·902	2·367
Calcium salts (weight in g.)	0·114	4·108	0·155	4·539	—	—
Volume of oxygen absorbed (cc.) ...	652	104	117	58	217	151
Respiration coefficient	4·4	15·4	12·8	15·4	6·2	9·7
Volume of hydrogen evolved (cc.) ...	392	Trace	Nil	Nil	262	196

purpose, in these preliminary experiments, has been to demonstrate the applicability of this method to the study of bacterial metabolism, and to cover a wide range of species or genera before selecting some of them for more detailed systematic study.

Table VIII.

	B. asiaticus-mobilis (Cast)	*B. asiaticus-mobilis* (Cast)	*B. alkalescens* (Andrewes)
Medium used	A	A	B
Catalogue number	C 70	C 70	C 73
Temperature	34°	34°	34°
Incubation period in days	2	2	41
Carbon balance sheet (g.)			
Carbon in solution (start)	4·912	4·912	5·191
„ added as $CaCO_3$	0·840	0·838	0·840
Total carbon (start)	5·752	5·750	6·031
Carbon in H_2SO_4	0·010	0·012	0·003
„ in CO_2	1·140	1·451	0·540
„ in bacteria	0·233	0·248	0·190
„ in residual $CaCO_3$	0·613	0·585	0·236
„ in solution	3·217	3·407	4·870
„ accounted for	5·213	5·703	5·839
„ accounted for (%)	90·6	99·2	96·8
Analysis of solution (g.)			
Carbon in residual glucose	0·013	0·000	1·960
„ in CO_2 in solution	0·006	0·006	—
„ in volatile acids	0·588	0·326	0·515
„ in non-volatile acids (Ca ppt.)	0·142	0·097	0·221
„ in volatile neutral compounds	0·941	0·984	0·122
„ in synthetic compounds	0·065	0·185	0·102
„ as butylene glycol	1·339	1·551	0·380
„ as lactic acid	Nil	Nil	1·300
Total carbon accounted for	3·094	3·149	4·600
Total carbon in solution	3·217	3·407	4·870
Carbon unaccounted for (by difference)	0·123	0·258	0·270
Residual glucose (%)			
Glucose (by polarimeter)	0·113	0·162	1·191
„ (by Shaffer-Hartmann)	0·007	Nil	0·980
„ (by Wood-Ost)	—	Nil	0·860
Acids			
Titration (calculated) (cc. *N*/1)	37·9	42·2	100·8
Volatile acids (cc. *N*/1)	8·54	6·49	17·4
Barium salts (weight in g.)	2·064	1·203	2·614
Calcium salts (weight in g.)	0·436	0·215	0·626
Volume of oxygen absorbed (cc.)	79	89	580
Respiration coefficient	21·6	25·1	—
Volume of hydrogen evolved (cc.)	308	430	Nil

Review of balance sheets.

The figures given in parentheses below are taken direct from the balance sheets and denote the weight of carbon, in the class of products mentioned, which is obtained from 5 g. of carbon as glucose. Hence the approximate yield % is obtained by multiplying the quoted figure by 20.

Leuconostoc. The species employed gives a high yield of lactic acid (1·008) and moderate yields of volatile acids (0·457) and of butylene glycol (0·311), considering that nearly half the original sugar remains unfermented.

B. aroideae and *B. carotovorus.* The balance sheets for these two species

Table IX.

	B. ureae Leube (*Urobacillus*)	*B. albo-lactis*	*Clostridium saccharo-butyricum*	*Clostridium pasteurianum*
Medium used	B	B	B (anaerobic)	B (anaerobic)
Catalogue number	C 72	C 98	C 96	C 94
Temperature	28°	28°	34°	34°
Incubation period in days ...	27	27	7	9
Carbon balance sheet (g.)				
Carbon in solution (start)	5·189	5·189	5·477	5·315
„ added as $CaCO_3$...	0·839	0·839	1·681	1·666
Total carbon (start)	6·028	6·028	7·158	6·981
Carbon in H_2SO_4	0·001	0·001	0·001	0·003
„ in CO_2	0·650	0·633	1·211	1·198
„ in bacteria	0·157	0·155	0·506	0·509
„ in residual $CaCO_3$	0·555	0·336	1·123	1·080
„ in solution	4·641	4·781	4·000	3·903
„ accounted for	6·004	5·906	6·841	6·693
„ accounted for (%)	99·6	98·0	95·6	95·9
Analysis of solution (g.)				
Carbon in residual glucose	2·486	1·840	1·346	1·538
„ in CO_2 in solution	0·005	0·006	0·006	0·006
„ in volatile acids...	0·287	0·532	1·892	1·744
„ in non-volatile acids (Ca ppt.)	0·184	0·081	0·104	0·121
„ in volatile neutral compounds	0·136	0·066	0·145	0·291
„ in synthetic compounds ...	0·061	0·075	0·109	0·126
„ as butylene glycol	0·841	0·062	—	—
„ as lactic acid	0·756	1·530	—	—
Total carbon accounted for	4·756	4·202	3·602	3·826
Total carbon in solution	4·641	4·781	4·000	3·903
Carbon unaccounted for (by difference)	0·115	0·579	0·398	0·077
Residual glucose (%)				
Glucose (by polarimeter)	0·911	0·563	0·583	0·664
„ (by Shaffer-Hartmann) ...	1·243	0·920	0·673	0·769
„ (by Wood-Ost)	1·206	0·900	0·664	0·710
Acids				
Titration (calculated) (cc. *N*/1) ...	47·3	83·8	93·0	97·7
Volatile acids (cc. *N*/1)	7·80	16·0	49·3	45·7
Barium salts (weight in g.)	1·070	2·590	12·51	10·65
Calcium salts (weight in g.)	0·537	Nil	0·162	0·216
Volume of oxygen absorbed (cc.) ...	430	470	Nil (anaerobic)	Nil (anaerobic)
Respiration coefficient	1·59	0·52	—	—
Volume of hydrogen evolved (cc.) ...	Nil	Nil	1263	1299

show considerable differences. *B. aroideae*, C 45, gives a very high yield of butylene glycol (2·136) equivalent to over 40 % of the original sugar, and very little lactic acid (0·288), whereas *B. carotovorus*, C 51, gives no butylene glycol and a high yield of lactic acid (1·152), corresponding to about 40 % on the sugar consumed.

B. proteus-vulgaris. In the first balance sheet the fermentation had not proceeded far although the incubation period was 6 days; therefore butylene

glycol and lactic acid were not determined. A second balance sheet experiment was performed in which the culture was incubated for 27 days. It was found that very little further fermentation had taken place, although the culture was still viable. With this organism, up to 30 % of the original sugar disappears in the early stages, while very little subsequent change is obvious. The early cessation of fermentation may be due to the exhaustion of one of the nutrients of the medium or to inhibition by products of bacterial metabolism.

B. acidi-lactici and *B. coli group*. This group is notable for the fact that it produces large amounts of lactic acid (up to 1·52) but negligible amounts of butylene glycol. A fair amount of volatile acid is produced (0·25–0·72), and a smaller amount of volatile neutral compounds (0·19–0·54). Too much importance should not be attached to the volatile acid determination, owing to the presence of large amounts of lactic acid. For the same reason, the "carbon unaccounted for" will tend to be low. The most interesting point is the fact that the "carbon unaccounted for" is not negligible; in two cases, it reaches the figures of 0·62 and 0·59 respectively, although 20 % of the original sugar still remains, and in spite of the fact that the "carbon unaccounted for" figure is probably too low, for the reason given above. These two species C 54 and C 37 are marked out for further investigation.

Aerobacter. This group produces lactic acid and butylene glycol. All grades are shown from a high yield of lactic acid (1·71) and a moderately low yield of butylene glycol to a low yield of lactic acid and a high yield of butylene glycol (1·60). In all cases, a fair amount of volatile neutral substances (probably ethyl alcohol) is formed (0·36–0·68). In no case is there any appreciable amount of "carbon unaccounted for". The balance sheets for *B. lactis-aerogenes* and *A. cloacae* bear out the results obtained by Harden *et al.* [1906; 1912, 1, 2].

The *B. asiaticus-mobilis* Castellani shows a remarkably rapid fermentation, the sugar in a 5 % solution being completely metabolised in less than 48 hours. The chief characteristic is the high yield of butylene glycol (1·34 and 1·55) which would make the organism specially suitable for the preparation of this glycol. A considerable amount of alcohol (0·94, 0·98) is formed, and a fair amount of volatile acid (0·59, 0·33). No lactic acid is produced, and the carbon unaccounted for is negligible.

A quantity of butylene glycol was prepared in the laboratory from the fermentation of glucose by *B. asiaticus-mobilis*. The fermentation was carried out in 750 cc. and 1 litre flasks containing 250 cc. and 350 cc. of medium respectively, also in 5 litre flasks containing 4 litres of medium. There was little difference in the yield obtained in the two cases, but the fermentation period was longer in the large flasks, due probably to the settling of the calcium carbonate with less rapid neutralisation of the acid produced. This effect was naturally less pronounced when the liquid was distributed in a shallow layer. Aeration had little or no effect on the fermentation, but as it provided a means of agitation of the carbonate it was advantageous from this point of view.

The best yields were obtained from a comparatively slow fermentation (7 days) occurring in presence of heavy carbonate and representing a 28–29 % recovery. No explanation is offered for this somewhat curious result.

The fermentation of 5 % sugar solution in small flasks occupied 2 days, but with higher concentrations of sugar longer periods were required—thus 12½ % sugar with increased amount of carbonate present needed 14 days for complete fermentation.

The method of recovery of the butylene glycol was very simple. The solution was filtered from undissolved calcium carbonate, evaporated to small bulk *in vacuo* and finally distilled *in vacuo*. Usually the solid residue remaining after distillation was washed with alcohol and a further small amount of the glycol recovered in this way. The total amount of butylene glycol recovered was 2435 g. representing an average yield of 22·3 % on the sugar fermented. The results of the experiments are summarised in Table X.

Table X. *Production of butylene glycol.*

Time in days	Initial sugar %	Initial sugar Weight in g.	Final sugar %	Final sugar Weight in g.	Sugar fermented g.	Glycol recovered g.	% yield	
5	5	25	0·21	1·0	24			
25	10	50	Nil	Nil	50	20	20·2	—
20	5	25	Nil	Nil	25			
4	5	1750	Nil	Nil	1750			
7	5	200	0·22	8·8	191	314	15·5	—
4	5	87·5	Nil	Nil	88			
6	5	1663·5	0·28	93·2	1570	450	28·6	Heavy $CaCO_3$
2	5	1750	0·45	157·5	1592	287	18·0	—
8	5	1200	0·40	96·0	1104	261	23·7	—
3	5	1750	Nil	Nil	1750	496	25·6	—
7	5	200	0·32	12·8	187			
7	5	440	Nil	Nil	440	126	28·6	Heavy $CaCO_3$
2	5	600	Nil	Nil	600	125	20·8	Aerated
4	5	400	0·22	17·6	382	87	22·8	Non-aerated
2	7·5	300	0·42	16·8	283	68	24·0	Aerated
6	10	400	Nil	Nil	400	89	22·3	Aerated
14	12·5	500	0·2	8	492	112	22·8	Aerated
				Total	10,928	2435	22·3	

B. alkalescens Andrewes, C 73. This organism produces a large amount of lactic acid (1·30), considerably smaller amounts of butylene glycol (0·38) and volatile acids (0·515). The carbon unaccounted for is small in amount.

B. ureae and *B. albolactis*. These two organisms show several points of interest. *B. ureae* (C 72) shows a small surplus in the balance sheet which is most unusual though possibly accounted for by the volatility of lactic acid. *B. albolactis* (C 98) on the other hand shows a "carbon unaccounted for" figure of 0·58, which is considerable in view of the amount of unfermented glucose remaining. The amount of "non-volatile acids" is low in both cases, but a fair amount of "volatile acids" is formed, particularly in the case of C 98. There is a curious discrepancy in both cases between the glucose determinations, the polarimeter figure being considerably lower than that obtained

by copper reduction. This appears to indicate the formation of some laevo-rotatory compound. *B. albolactis* thus merits further investigation.

Clostridium. These anaerobic species are remarkably similar in behaviour. The characteristic is the production of a large amount of volatile acids (1·89 and 1·74). Butylene glycol and lactic acid were not determined, but could only be present in small amount, since the "carbon unaccounted for" is low.

Discussion.

In performing these balance sheet experiments the time allotted for each fermentation varies since the time was chosen according to the rate of fermentation. An endeavour was made in each case to interrupt the experiment at such a point that most, but not all, of the sugar had disappeared, so that the amount of intermediate metabolic products should be as large as possible. This involves wide differences in the incubation periods of the various organisms. In cases where the fermentation is slow, there is a greater likelihood of bacterial autolysis. This introduces the possibility of carbohydrate breakdown or attack on the intermediate products by the enzymes liberated by cell autolysis, in addition to the coordinated fermentative action of these enzymes when under the control of the living cell. This difficulty cannot be entirely obviated in work of this type, but it would be advisable in future experiments to determine growth curves for each organism studied, in order to correlate the fermentation during the rapid growth period with that occurring during the autolysis period, as indicated by a decline in the viable count.

The possibility of autolysis raises an interesting point in the interpretation of the balance sheets. The "carbon in bacteria" will not represent the total amount of carbon utilised to form cell-matter if a portion of the cells autolyses in the later stages of fermentation. However, it appears likely that a large proportion of the autolysis products will, in such cases, be found in the "carbon in synthetic products", *i.e.* in the carbon compounds precipitated by "colloidal iron."

It has been shown that the quantitative method for the classification of the types of metabolic products of micro-organisms although hitherto used only for moulds, applies equally well to bacteria provided certain minor modifications are made. The chief difficulty consists in finding a suitable synthetic medium containing no carbon apart from the glucose supplied for fermentation. Although some of the organisms were able to grow and ferment on such a medium, the number was found to be very limited, and a much wider field became available by the introduction of small amounts of organic nitrogen in the form of peptone. This is justifiable so long as the amount of carbon thus introduced is small in relation to the carbon present as glucose.

Reference has already been made to the fact that addition of calcium carbonate to the medium was found more generally useful as a method of neutralising the acidity formed than the use of large amounts of phosphate.

In the case of two organisms, *Aerobacter aerogenes* and *cloacae* (C 24 and C 12), comparative balance sheets were obtained using the carbonate and the phosphate buffer media. A study of these reveals the fact that although fermentation was retarded on the phosphate medium, the products were not widely different in nature, the chief difference being a considerable increase of the non-volatile acid fraction with excess of phosphate. This may be due to the formation of hexosephosphate. In a third case—that of *B. acidi-lactici* (Hueppe), C 6, on the phosphate medium practically no gas was liberated during the 6 days of the experiment, only about half the sugar had disappeared and at the end of the period the organism was apparently dead. The balance sheet is not given. On the carbonate medium, the fermentation was definitely faster and was still in progress at the end of 6 days. The phosphate buffering appears inadequate when the organism produces a large amount of acid, as in the case of C 6.

Many of the bacteria appear to utilise glucose merely for growth purposes. This is shown by the large number of control experiments in which only a small proportion of the sugar disappeared. These organisms cannot therefore be said to ferment the sugar in the usual sense of the term.

We may now compare briefly the types of products obtained from bacteria with those derived from moulds. The mould products (apart from mycelium and carbon dioxide) group themselves chiefly in the classes "volatile neutral substances" (ethyl alcohol), "non-volatile acids" and "carbon unaccounted for", the last named, when large in amount, often consisting of mannitol or glycerol. Only traces of volatile acids are produced. Lactic acid and butylene glycol have never been reported as mould metabolic products. With moulds, on the other hand, various synthetic substances such as polysaccharides, sterols, pyrones, quinonoid and phenolic substances, complex fatty acids, *etc.*, are formed.

The main products of bacterial metabolism are "volatile neutral substances", "volatile acids" and much smaller amounts of "non-volatile acids". Lactic acid and butylene glycol are of frequent occurrence and are often produced in considerable quantity. Thus species of *Aerobacter* give varying amounts of both products, of *Escherichia* give considerable amounts of lactic acid but little butylene glycol, and of *B. asiaticus mobilis* (Cast.) large amounts of butylene glycol but no lactic acid.

Hydrogen is a frequent product of bacterial action, particularly in the case of anaerobes, but has never been obtained from moulds.

Although polysaccharides are known to be formed [Grey, 1924], no evidence has yet been obtained of any phenolic substances such as are elaborated by moulds.

The products of bacterial metabolism have been shown to be, in general, more volatile than those of moulds. Thus, volatile acids replace the non-volatile acids, butylene glycol is obtained instead of mannitol or glycerol, and so on. This may be due to the fact that moulds are obligate aerobes, whilst many bacteria are facultative anaerobes, and consequently mould

metabolic products are more highly oxidised. It is well known that an increase in the oxygen content of organic substances (as hydroxyl or carboxyl groups) lowers their volatility. In this connection it is interesting to note [Hermann and Neuschul, 1931] that eight strains of acetic acid bacteria, the reactions of which are almost purely oxidative and only feebly hydrolytic, give products such as gluconic acid, substances which are more akin to those of mould metabolism.

The oxidative nature and greater synthetic activity of mould fermentation may be said to be the chief characteristics which differentiate it from bacterial fermentation.

SUMMARY.

The Czapek-Dox medium as used for the determination of mould metabolism products by the balance sheet method has been modified so as to render it suitable for the cultivation of bacteria. This involves the substitution of the sodium nitrate of the original medium by microcosmic salt of equivalent nitrogen content. The number of organisms showing growth and fermentation on this medium was small but was greatly increased by addition also of 0·2 % peptone, an amount insufficient to interfere appreciably with the carbon balance sheet estimations. Calcium carbonate proved to be the most useful buffering agent. Certain modifications necessary when applying the standard balance sheet technique to bacteria are detailed, and methods employed for the determination of 2 : 3-butylene glycol and lactic acid, which are of frequent occurrence, are described.

Fifty species of bacteria were found to grow readily on the selected medium and of these, 20 species were chosen for examination by the balance sheet method at 28° and 34°. The balance sheets show that the chief classified products are volatile acids, volatile neutral substances, lactic acid and 2 : 3-butylene glycol.

Of these organisms, *B. asiaticus-mobilis* proved interesting in that it gave about a 30 % yield of butylene glycol and the rate of fermentation was faster than for any of the other organisms examined, whilst the balance sheets of three other organisms (*B. acidi-lactici*, C 54, *Esch.* species from milk, C 37, and *B. albolactis*, C 98) indicate that these species merit further investigation.

We greatly appreciate the interest taken in this work by Professors H. Raistrick and W. W. C. Topley, and our best thanks are due to them for their advice and helpful criticism. We are also indebted to Mr W. Rintoul and the Directorate of Imperial Chemical Industries for permission to publish this work.

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CLXVII. ON THE GLYOXALASE-CO-ENZYME RATIO IN LIVER TISSUE IN INANITION[1].

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In a previous communication [Vogt-Møller, 1931] on the glyoxalase-co-enzyme ratio in liver tissue in avitaminosis $B_1 + B_4$ [Reader, 1930], *i.e.* in experimental beriberi in mice, it was stated that starved animals, from which food was withdrawn, but which were allowed to take water, differed from animals suffering from avitaminosis $B_1 + B_4$, whose allowance of food was complete in every respect with the exception of vitamins B_1 and B_4, in such a manner that the liver tissue of the former animals (and probably the whole organism except the pancreas, in which special conditions prevail) in contradistinction to that of the latter contained more co-enzyme (co-mutase), though in both cases less than the normal amount was present. Under the conditions of the experiments, therefore, methylglyoxal was partly stabilised in avitaminous ferment systems.

When the technique that was then used is employed, *viz.* the determination of acidity described by Neuberg [1913, 1, 2] and by Dakin and Dudley [1913, 1, 2, 3], the precipitation of methylglyoxal employed by Vogt [1929], either as *p*-nitrophenylosazone or as *bis*-2 : 4 dinitrophenylhydrazone (described by Neuberg and Kobel [1928]), and the colorimetric method described by Ariyama [1928], all the methods being employed after 24 (48) hours' incubation of the ferment system at 37°, and when the technique used by me for the institution of inanition is used, *i.e.* the food but not the water is removed from the cage, the floor of which is covered with wood-wool and sawdust, the facts previously stated still hold good, provided the inanition is not carried too far.

Having begun to use Ariyama's method, but only with 20 minutes' incubation, and letting the animals starve in glass cages devoid of shavings instead of in the manner referred to above, I have attained results which deviate somewhat from those previously communicated, for which reason they are now briefly published.

As to the quantitative (colorimetric) determination of methylglyoxal I refer to Ariyama's communication. The incubation time was only 20 minutes at 37°, and by determining the amount of the added methylglyoxal which has been used during this period a measure of the glyoxalase + co-enzyme

[1] Supplementary note to my article in *Biochem. J.* (1931), **25**, 418.

content of the organ (in this case of the liver) is obtained. As glyoxalase + co-enzyme preparation I used a 20 % preparation of ground liver tissue suspended in distilled water after it had been allowed to stand for 1 hour at room temperature after evisceration and after filtration of the suspended tissue. As co-enzyme preparation I used a 10 or 20 % boiled (5 minutes) preparation of suspended liver tissue prepared as stated above. Co-enzyme-free preparations were prepared by means of 6 hours' dialysis of the glyoxalase + co-enzyme preparations.

Using this method it appeared that an inanition of short duration (42 hours in the case of mice) involved a very considerable loss of the co-enzyme content of the liver tissue. It is a well-known though remarkable fact that the co-enzyme is used up during the fermentation process *in vitro*; the same thing may also occur *in vivo*. In contradistinction to this fact Ariyama found that after 10 days' inanition the amount of glyoxalase (*i.e.* glyoxalase + co-enzyme) in liver tissue (rabbit) "remained unchanged," whilst Sakuma [1930] found that 10 days' inanition of a rabbit caused a considerable decrease of the amount of co-enzyme.

In relation to what has been communicated previously, *viz.* the abnormal conditions of carbohydrate metabolism in avitaminosis B_1, I tried, in relation to the glucose cure [Kinnersley *et al.* 1928], the possible co-enzyme-forming or co-enzyme-stimulating power of glucose (saccharum amylaceum puriss. anhydric. U.S.P.X.) and found, like Sakuma, that weak solutions of glucose *in vitro* furthered the dismutation of methylglyoxal in dialysed, *i.e.* co-enzyme-free, ferment systems, and similar conditions were also found in the case of weak solutions of sodium nucleinate (originating partly from yeast, partly from the thymus gland). This fact in view of Euler and Myrbäck's [1929] supposition that co-enzyme (co-mutase) is "ein Adenin-nucleotid besonderer Art" brings the co-enzyme in a rather close relation to vitamin B_1 or perhaps especially vitamin B_4, without, however, allowing us to identify these two substances. One might suppose that the vitamin contained constituents necessary for the composition or formation of the co-enzyme. This much is certain: in inanition and in avitaminosis $B_1 + B_4$ there is a decrease of the amount of co-enzyme, a decrease that is not found in the controls used (paired-feeding method). It is possible that the cause of the decrease of the co-enzyme concentration in avitaminosis $B_1 + B_4$ should be looked for in the anorexia, always engendered by the avitaminosis, although this is not very probable when the conditions of the controls are considered, but it is now at least within our reach to find out the substances (nutritives, chemical compounds) causing the formation of co-enzyme *in vivo*. Possibly the toxic effect of glucose injected parenterally in excess is best understood on the ground that by the catabolism of glucose the tissue is deprived of the co-enzyme.

SUMMARY.

Information is given about the decrease of the co-enzyme concentration of liver tissue in inanition and about the co-enzymic properties of glucose and sodium nucleinate. Attention is drawn to the fact that it is now possible to examine the co-enzyme formation *in vivo*.

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CLXVIII. THE REDUCTION OF NITRATES BY *BACT. COLI.*

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Introduction.

It has long been known that many bacteria are able to reduce nitrates to nitrites, and this fact is extensively used in classification. More exact investigation of the process is of recent date. Quastel, Stephenson and Whetham [1925] showed that nitrate could produce anaerobic growth of some facultative anaerobes in media which without it would not support growth, *i.e.* the nitrate could take the place of molecular oxygen. The coupled reaction, with reduction of nitrate to nitrite, always yields less energy than the corresponding direct oxidation, *e.g.*,

$$\text{lactic acid} + KNO_3 \rightarrow \text{pyruvic acid} + H_2O + KNO_2 + 30{\cdot}7 \text{ cals.}$$
$$\text{lactic acid} + \tfrac{1}{2}O_2 \rightarrow \text{pyruvic acid} + H_2O + 51{\cdot}9 \text{ cals.}$$

Quastel and Whetham [1924] showed that the utilisation of nitrate as a hydrogen acceptor depended on the power of the organism to activate the nitrate molecule, by demonstrating that nitrates oxidise leucomethylene blue in the presence of a washed suspension of cells but not in its absence. The capacity of a species of bacteria for anaerobic growth on, say, lactate and nitrate, is, of course, parallel to its power of oxidising leucomethylene blue by nitrate.

In its rôle as a supporter of growth in media containing only non-fermentable carbon compounds nitrate is strictly comparable with molecular oxygen, and the object of the work described here is to make a comparison between the reduction of nitrate to nitrite and that of oxygen to water.

In considering the enzymic mechanisms involved, there appear to be three possibilities, *viz.*,

(1) the activations of oxygen and nitrate are carried out by the same enzyme;

(2) the enzymes concerned are different, and specific for oxygen and nitrate respectively;

(3) the activation of nitrate is carried out by an accessory mechanism in

[1] Goldsmiths' Senior Student and Benn W. Levy Student.

close connection with the oxygen-activating enzyme. This last is a very vague conception, which may have to be considered if the other hypotheses fail.

Methods.

The organism chosen was *Bact. coli* (Escherich); two different preparations of the cells were used, first an ordinary washed and aerated suspension, and second the same suspension treated with toluene at room temperature [Quastel and Wooldridge, 1927; Cook, 1930]. For different purposes the one or the other proved more convenient, but wherever the same experiment could be carried out with both, essentially similar results were obtained. The chief differences of behaviour produced by toluene treatment are (1) the endogenous respiration of the cells is reduced to negligible proportions, *i.e.* methylene blue, oxygen and nitrate are not reduced except in the presence of an added donator such as hydrogen, formate, lactate, *etc.*, (2) the oxidation of lactate and succinate proceeds only as far as pyruvate and fumarate respectively, and (3) the suspension is incapable of attacking glucose.

The course of the reduction of nitrate was followed by two methods. The first consisted of placing the cell suspension in the cups of a Barcroft differential manometer, with a measured amount of nitrate in the right-hand cup, filling the apparatus with hydrogen, and observing the rate at which hydrogen was absorbed [*cf.* Stephenson and Stickland, 1931]. The other method was carried out by keeping the suspension anaerobic in the presence of a known concentration of nitrate and of hydrogen donator (*e.g.* lactate) and estimating the nitrite produced, at various time intervals, colorimetrically by means of the Griess-Ilosvay reagent. The most convenient apparatus for this purpose was the ordinary Thunberg tube. A set of tubes containing nitrate, donator (usually lactate), buffer and cell suspension was evacuated and placed in a bath, and for each reading one tube was taken out and the concentration of nitrite in it estimated.

To prepare the reagent completely colourless the sulphanilic acid and α-naphthylamine were recrystallised from water and light petroleum respectively, dissolved in 50 % acetic acid made with glass-distilled water, and mixed in equal proportions when required. Glass-distilled water was used in making up all solutions, as the ordinary distilled water often contained appreciable amounts of nitrite. The appropriate quantity of the solution to be estimated was added to 5 cc. of the reagent, and made up to 20 cc. with 50 % acetic acid. The standard was a 0·0001 *M* solution of pure recrystallised silver nitrite, of which 1 or 2 cc. were used. The maximum colour developed in 15–20 minutes and was stable for at least 24 hours. By this method duplicate tubes always agreed to within 3 %. All the experiments were carried out at 40° in the presence of phosphate buffer at p_H 7·0, except where otherwise stated.

Preliminary observations.

(1) *The extent of reduction of nitrate.* With toluene-treated cells, using the Barcroft manometer method, the hydrogen taken up corresponded to 90–100 % of the theoretical for reduction to nitrite, *e.g.*

	Amount of KNO_3	H_2 uptake mm.³	Theoretical	%
1	0·5 cc. *M*/50	227	224	101
2	,,	210	224	94
3	,,	215	224	96

Other donators, added in excess to known amounts of nitrate in Thunberg tubes, with either normal or toluene-treated suspensions, also gave 100 % reduction as measured by nitrite estimations, *e.g.* with lactate,

	KNO_3 added *M*	Nitrite found *M*	%
1	0·0250	0·0245	97·5
2	0·0200	0·0197	98·5
3	0·0200	0·0198	99

With untreated suspensions, the hydrogen uptake by the first method always fell short of the theoretical, owing to reduction of part of the nitrate by other donators in the cells [see Stephenson and Stickland, 1931], but estimation of nitrite showed the reduction to be complete.

(2) *The extent of oxidation of hydrogen donators.* Cook and Stephenson [1928] showed that with oxygen as the hydrogen acceptor an untreated suspension of *Bact. coli* oxidised formate completely, while with lactate the oxygen uptake was two-thirds of that required for complete oxidation, and with succinate there was a rapid oxidation as far as fumarate with an indefinite and slower further oxidation. It has been impossible to get parallel results with nitrate, owing to the great rapidity with which the untreated cells reduce nitrate in the absence of added donators.

In the case of toluene-treated suspensions, Quastel and Wooldridge [1927] and Cook [1930] have shown that formate is completely oxidised in oxygen,

Donator	Conc. of donator *M*	Conc. of NO_2^- *M*	Extent of oxidation %	Average %	O_2 uptake (Cook) %
Lactate	0·004	0·00332	83		
		0·00353	88		
		0·00317	79	82	78
	0·002	0·00159	80		
		0·00162	81		
Succinate	0·004	0·00312	78		
		0·00337	84		
		0·00303	76	82	82
	0·002	0·00168	84		
		0·00178	89		
Formate	0·0051	0·00472	93		
		0·00461	90		
		0·00472	93		
		0·00465	91	92	98
	0·00255	0·00238	93		
		0·00231	91		

while lactate and succinate go as far as pyruvate and fumarate respectively. The results for reduction of nitrate were obtained by incubating tubes containing a measured amount of the donator and excess of nitrate until there was no further increase in the nitrite concentration. The results are given above, with Cook's [1930] figures for the percentage of the theoretical oxygen uptake in the last column. The oxidations are incomplete, but proceed as far as they do with oxygen.

(3) *The linearity of the reaction.* Starting with 0·02 *M* potassium nitrate and 0·02 *M* lactate, and using a dilute suspension of cells, the initial course of the reaction may be determined while less than 1 % of the reaction is taking place, and the rate was found to be constant within the limits of accuracy of the method (Fig. 1). With a thicker suspension the whole course of the reaction may be followed, as in Fig. 2.

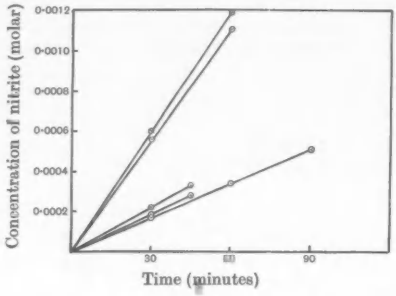

Fig. 1.

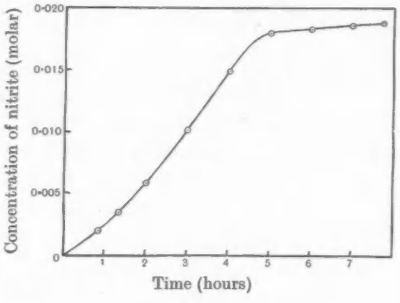

Fig. 2.

(4) *The affinity of the enzyme for nitrate.* The initial velocity of the reaction was measured at a series of nitrate concentrations by the vacuum tube method. No great decrease in velocity with diminishing concentration was observed until concentrations of nitrate were reached at which the method of estimation

of the nitrite had reached its limit, so the lowest points on the curve (Fig. 3) are not very accurate; it was possible, however, to make an estimate of the

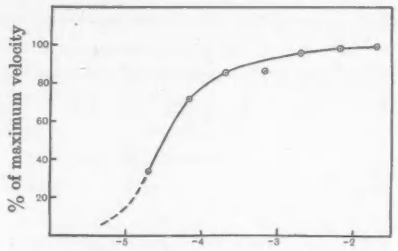

Fig. 3.

affinity, and the three separate values obtained for the Michaelis constant were 0·00005, 0·00003 and 0·00005 *M*. Hence one can be sure of being able to get initial velocity values at concentrations of nitrate down to at least 0·0005 *M*.

(5) *The effect of* p_H *on the velocity.* Using the vacuum tube method for measuring the rates, the curves shown in Fig. 4 were obtained, giving the

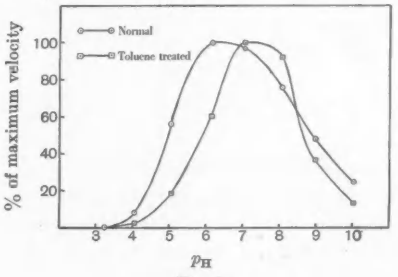

Fig. 4.

relation between p_H and initial velocity of nitrate reduction. Treatment with toluene causes a marked change in the p_H curve. Both curves are given as percentages of the maximum velocity, but the actual optimum with the toluene-treated suspension is only about 60–70 % of that before treatment, so that the apparent acceleration at p_H 8 is not real.

(6) It was also shown that variation of the lactate concentration within narrow limits about 0·02 *M* caused no great difference in the reaction velocity:

Lactate conc. (*M*)	Initial velocity (*M* NO_2^- per hour)
0·02	0·00081
0·0067	0·00079
0·002	0·00069

As a consequence of these preliminary results, the following was used as a standard method.

1 cc. 0·1 *M* potassium nitrate, 1 cc. 0·1 *M* sodium lactate, 1 cc. phosphate buffer p_H 7·0, 1 cc. water and 1 cc. of the suspension of cells were placed in each Thunberg tube, and the tubes were evacuated and placed in a water-bath at 40°. At 30 and 60 minute intervals tubes were removed and the nitrite in them was estimated, the concentration of cells being adjusted so that after 30 minutes roughly 1 % of the nitrate was reduced. The two points almost always lay on a straight line through the origin, and experiments showing any significant departure from linearity were discarded.

The effect of cyanide on nitrate reduction.

Most biological oxidations involving the use of molecular oxygen are strongly inhibited (though not always completely) by low concentrations of cyanide of the order of 0·0001–0·001 *M*. In the case of *Bact. coli* it has been shown [Cook, Haldane and Mapson, 1931] that the inhibition of the oxidation of formate, lactate and succinate tends towards 100 % as the cyanide concentration is increased.

The inhibitory effect of cyanide on nitrate activation may be shown qualitatively in the following simple way. Four sets of vacuum tubes containing the following solutions

	Buffer cc.	Methylene blue cc.	KNO_3 *M*/40 cc.	KCN *M*/200 cc.	Water cc.	Bact. susp. cc.	Reduction time at 40° min.
1	1	1	—	—	2	1	7·75
2	1	1	1	—	1	1	∞
3	1	1	—	1	1	1	5·25
4	1	1	1	1	—	1	8

were evacuated and filled with hydrogen, with the usual precautions for preventing loss of hydrogen cyanide. The reduction times show that whilst nitrate prevents the reduction of methylene blue, this inhibition is almost completely removed by cyanide.

Quantitatively each of the two methods described has been applied to the measurement of the inhibition, with similar results. With the Barcroft manometer method special precautions must be taken to prevent loss of hydrogen cyanide, either in filling the cups with hydrogen or by absorption in the potash which usually occupies the small tubes inside the cups. The former difficulty was overcome by thoroughly evacuating the solutions used before placing them in the apparatus, to remove dissolved oxygen, and then partially evacuating and filling with hydrogen several times until the amount of air left was negligible; the latter by omitting the potash from the cups. This was justified by the fact that no carbon dioxide can be produced in the reaction and also by direct comparison of experiments with and without potash. In this way the velocity of hydrogen uptake at various concentrations of potassium cyanide was compared with that in the absence of cyanide.

The results with lactate as donator were obtained by using vacuum tubes with hollow stoppers to contain the cyanide solution, which was mixed with the rest of the contents of the tube after evacuation; the alkalinity of the potassium cyanide solution was sufficient to prevent appreciable loss of the acid during evacuation.

The results of all the measurements are given in Fig. 5. Toluene treatment

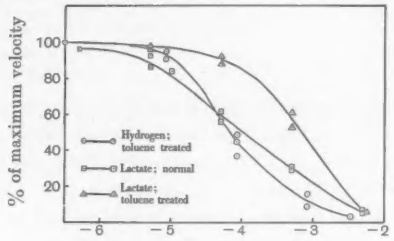

Fig. 5.

of the suspension has a considerable effect on the inhibition of nitrate reduction by cyanide, making the enzyme more susceptible to the reagent.

The extent of the inhibition does not depend on the nitrate concentration, *i.e.* it is as usual non-competitive.

KCN *M*	KNO_3 *M*	Velocity (*M* NO_2^-/hr.)	Velocity of control	Inhibition %
0·00002	0·02	0·000043	0·000114	62
0·00002	0·001	0·000035	0·000101	65

The effect of carbon monoxide.

The Barcroft manometer method of measuring the velocity of reduction cannot be applied in this case, as carbon monoxide has an inhibiting effect on hydrogenase which would interfere; the following figures show the effect of about half an atmosphere pressure of carbon monoxide on the rate of reduction of methylene blue by hydrogen in vacuum tubes with a toluene-treated suspension of *Bact. coli*:

Hydrogen mm.	Carbon monoxide mm.	Reduction time (mins.)
200	0	7·5
180	325	18·75

The lactic dehydrogenase is entirely unaffected by carbon monoxide [Cook, Haldane and Mapson, 1931], so the vacuum tube method can be applied; this is one of the reasons why lactate was chosen as the standard donator in this work.

Wherever carbon monoxide inhibits respiration, the inhibition is found to be a competitive one, so if the analogy between oxygen respiration and nitrate

respiration is to be borne out the inhibition by carbon monoxide should be greatest at the lowest nitrate concentrations. Neither at high or low nitrate concentrations was any inhibition observed, *e.g.*

KNO_3 (*M*)	CO (mm.)	Velocity (*M* NO_2^-/hr.)
0·02	0	0·000431
	117	0·000438
	325	0·000433
0·002	0	0·000384
	120	0·000410
	315	0·000391
0·0002	0	0·0000270
	650	0·0000265

It is essential in these measurements to find the initial velocity, as any change in the nitrate concentration would produce a double error, partly due to the assumed increase in inhibition by carbon monoxide at the lower nitrate concentration, and partly to the fact that the enzyme is not saturated with its substrate at 0·0002 *M*, so that the velocity would fall off as the concentration decreased. The lowest nitrite concentration that can be estimated with reasonable accuracy by the method employed here is 0·00001 *M*, so that if anything like an initial velocity is to be measured the initial concentration of nitrate must not be taken below, say, 0·00004 *M*.

Using this minimum concentration of nitrate, several experiments have been carried out. 3 cc. of each of 0·0002 *M* potassium nitrate, 0·1 *M* sodium lactate, phosphate buffer at p_H 7·0 and water were measured into each of six 100 cc. Büchner flasks, and 3 cc. of cell suspension added at such a dilution that about 0·00001 *M* nitrite would be expected in 30 minutes. The flasks were evacuated and one-half of them filled with carbon monoxide purified from oxygen. After 30 minutes' immersion in the water-bath, with vigorous shaking at first to ensure saturation with the gas, they were taken out and 10 cc. of their contents used for nitrite estimations. Typical results were

		Velocity (*M* NO_2^-/hr.)
1	Without CO	0·0000164
	With CO (about 1 atmosphere)	0·0000160
2	Without CO	0·0000212
	With CO (about 1 atmosphere)	0·0000203

These differences are not outside the experimental error, so the conclusion is that at a concentration of nitrate of 0·00004 *M* one atmosphere pressure of carbon monoxide (about 0·0007 *M* CO in solution) produces no measurable inhibition.

The effect of oxygen.

Before this subject could be investigated, a few preliminary results were required. First, it was shown that *Bact. coli* cannot re-oxidise nitrite to nitrate by means of oxygen. Tubes containing buffered 0·00025 *M* sodium nitrite and a thick cell suspension were aerated with a current of air saturated with water vapour, and samples were withdrawn for estimation of nitrite. No re-oxidation was observed.

Time (mins.)	$NaNO_2$ (*M*)
0	0·000246
30	0·000248
60	0·000260
140	0·000247

Second, the rates of reduction of nitrate in the absence of oxygen and of oxygen in the absence of nitrate were compared directly. It was found that, taking one atom of oxygen as comparable with one molecule of nitrate, oxygen is reduced about ten times as fast as nitrate, *e.g.*

Normal suspension	0·0221 g. atoms O_2 per hour 0·00236 g. mols. KNO_3 per hour
Toluene-treated suspension	0·0034 g. atoms O_2 per hour 0·000352 g. mols. KNO_3 per hour

To investigate the inhibition of nitrate reduction by oxygen quantitatively, it was necessary to vary the concentration of oxygen used, and this was done by diluting air with nitrogen and bubbling the mixtures through the solutions. The details of the method employed are as follows. A large aspirator was filled with the gases in roughly the right proportions, and the oxygen in the mixture estimated. 2 cc. of 0·1 *M* sodium lactate, phosphate buffer at p_H 7·0, water and nitrate solution were measured into each of a series of large test-tubes with corks, connected with pressure tubing, and a rapid stream of the gas bubbled through for about 15 minutes to saturate the solutions with oxygen at the right pressure. Then 2 cc. of the suspension of bacteria were added, and the bubbling was continued while samples were withdrawn for estimation of nitrate at various intervals. The velocity of nitrate reduction *in vacuo* was determined simultaneously. To make sure that at the lowest oxygen pressures no appreciable part of the oxygen was being removed from the gas, a sample was collected in one experiment as it issued from the last tube, and analysed. Before passing through the tubes the gas gave duplicate analyses of 0·42 % and 0·41 % oxygen, and after, 0·41 % and 0·44 %.

A series of results was obtained with nitrate concentrations of 0·02 *M* and 0·002 *M*; the extent of the inhibition was identical in the two cases, so only one set of figures (those for 0·02 *M*) is given. These results are plotted in Fig. 6, the concentrations of oxygen being given logarithmically.

O_2 %	O_2 in sol. *M*	Velocity	Velocity of control	Inhibition %
99	0·00100	0·000046	0·00120	96
20·9	0·00021	0·000026	0·000513	94
3·76	0·000038	0·000026	0·000387	93
1·65	0·000017	0·000047	0·000412	88
1·09	0·000011	0·000136	0·000352	61
0·42	0·0000042	0·000259	0·000350	26
0·36	0·0000036	0·000446	0·000568	21

For reasons which will be explained in the discussion of these results, the effect of carbon monoxide on the inhibition of nitrate reduction by oxygen was investigated. Experiments similar to those already described were done

with mixtures containing different amounts of oxygen with roughly ten times as much carbon monoxide. The inhibition was in each case much decreased (see Fig. 6).

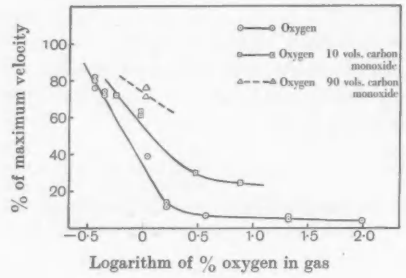

Fig. 6.

O_2 %	CO %	CO/O_2	O_2 in sol. *M*	CO in sol. *M*	Velocity	Velocity of control	Inhibition %
7·62	84·3	11·1	0·000077	0·00063	0·000124	0·000508	75·5
3·00	31·9	10·6	0·000030	0·00024	0·000132	0·000440	70
0·94	9·8	10·5	0·0000095	0·000073	0·000227	0·000370	39
0·55	4·5	8·9	0·0000056	0·000033	0·000398	0·000528	24
In addition one experiment was done with a larger CO/O_2 ratio:							
1·03	91·2	89	0·0000104	0·00068	0·000296	0·000391	24

A further question of importance is whether the extent of inhibition by oxygen depends on the concentration of nitrate. As already stated, initial concentrations of nitrate of 0·02 *M* and 0·002 *M* gave identical results, in the presence or absence of carbon monoxide, at all concentrations of oxygen between 0·000004 *M* and 0·001 *M* (0·4 % and 99 % of an atmosphere). The following results, obtained in the same way, show that the inhibition is also the same with 0·0002 *M* nitrate, a concentration at which the velocity of reduction is already falling off owing to unsaturation of the enzyme.

O_2 %	O_2 in sol. *M*	KNO_3 *M*	Velocity	Velocity of control	Inhibition %
0·36	0·0000036	0·02	0·000446	0·000568	21
,,	,,	0·002	0·000416	0·000547	24
,,	,,	0·0002	0·000332	0·000402	18

A few experiments were carried out to see if nitrate affects the velocity of reduction of oxygen. Two pairs of Barcroft manometers were set up, one pair with 0·02 *M* sodium lactate, the other with 0·02 *M* lactate and 0·01 *M* nitrate, both in air. The nitrate seemed to slow down the oxygen uptake about 5 %, but this is not really outside the experimental error, and cannot be considered significant.

Discussion.

There is no difficulty in deciding between the three possibilities mentioned in the introduction, with regard to the enzymes concerned in the activation of oxygen and of nitrate.

First, the relative affinities of the enzymes for their substrates and for carbon monoxide show that two entirely different mechanisms are concerned. Cook, Haldane and Mapson [1931] state that oxidations by oxygen are still working at their maximum velocity in oxygen at 4 % of an atmosphere pressure, *i.e.* at 0·00004 *M*, so that the Michaelis constant of the oxygen enzyme for its substrate is at any rate less than 0·000004 *M*; and they showed that the affinity of the enzyme for carbon monoxide is one-tenth that of its affinity for oxygen (when lactate is the hydrogen donator), so that the Michaelis constant of carbon monoxide for the oxygen enzyme is at any rate less than 0·00004 *M*. In the case of nitrate, the Michaelis constant for the substrate is about 0·00005 *M*, while carbon monoxide (0·0007 *M*) causes no inhibition in the presence of 0·00004 *M* nitrate, so that the Michaelis constant of the nitrate enzyme for carbon monoxide must be at any rate greater than 0·007 *M*. The values $< 0{\cdot}00004$ and $> 0{\cdot}007$ are sufficiently divergent to show that the two enzymes cannot be the same.

Second, if the same enzyme activated both oxygen and nitrate, the inhibition of the reduction of one substrate caused by the presence of the other should be competitive, whereas the results show that the amount of inhibition at any oxygen pressure is independent of the nitrate concentration. Thus the facts can be accounted for only by assuming two separate specific enzymes.

There remains to be explained the fact that oxygen causes a non-competitive inhibition of nitrate reduction, and a simple and qualitatively satisfactory explanation will now be given. Lactic dehydrogenase is providing a constant supply of activated lactic acid, and this, or the intracellular hydrogen carriers reduced by it, is shared between the oxygen and the nitrate enzymes. When both these enzymes are saturated with their substrates, the relative amounts reduced will depend on the "concentrations" of the enzymes and on various other factors. Considering the facts that oxygen is reduced ten times as fast as nitrate, and that the maximum inhibition of nitrate reduction by oxygen is 96 %, it will be seen that under these conditions 0·4 % of the available lactate is oxidised by nitrate and 99·6 % by oxygen. If now the rate of activation of oxygen is decreased by lowering the oxygen pressure, a greater proportion of the lactate will be oxidised at the nitrate enzyme; *e.g.* at 0·36 % oxygen, giving 20 % inhibition of the nitrate reduction, 8 % of the available lactate is oxidised by nitrate, 92 % by oxygen. Similarly, if the activation of oxygen is inhibited by carbon monoxide, a greater proportion of nitrate will be reduced; a CO/O_2 ratio of ten, giving 50 % inhibition of oxygen uptake, reduced the limiting inhibition from 96 % to about 80 %, so that 2 % of the lactate was oxidised by nitrate and 98 % by oxygen. It can be seen that nitrate cannot alter the rate of oxygen uptake sufficiently to give significant results on a Barcroft manometer apparatus.

Though the system is too complicated for exact mathematical treatment, the facts are qualitatively explained by this view.

In its behaviour with cyanide the nitrate-activating enzyme is similar to

other oxidising enzymes such as indophenol oxidase and peroxidase; with *Bact. coli*, using lactate as hydrogen donator, 50 % inhibition of the oxygen-activating enzyme is produced by 0·001 *M* cyanide, and 50 % inhibition of the nitrate enzyme by 0·0001 *M*. The inhibition is non-competitive in both cases [Cook, Haldane and Mapson, 1931]. The lack of inhibition by carbon monoxide is not unusual; Dixon [1928] has shown that the catalysis of the oxidation of cysteine by haematin is not inhibited by this reagent.

SUMMARY.

1. *Bact. coli* reduces nitrate quantitatively to nitrite. After toluene treatment it oxidises formate, lactate and succinate to the same degree by means of nitrate as it does with oxygen, *i.e.* to carbon dioxide, pyruvate and fumarate respectively.

2. The reduction can be completely inhibited by cyanide, 50 % inhibition being produced by about 0·0001 *M* KCN.

3. As far as the method used allowed, no inhibition by carbon monoxide could be observed.

4. Oxygen causes a non-competitive inhibition of nitrate reduction; this inhibition is partly removed by carbon monoxide.

5. A theory is offered which explains these results qualitatively.

I wish to record my thanks to Miss Stephenson, Drs Dixon and Woolf, and Prof. J. B. S. Haldane for useful advice, and to Sir F. G. Hopkins for much help and encouragement.

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CLXIX. STRUCTURE AND ENZYME REACTIONS.

X. ACTION OF SALTS ON THE SYSTEMS: AMYLASE-STARCH-PROTEINS.

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In the present paper the results obtained from a study of the effect of addition of electrolytes (NaCl, $MgCl_2$, $CaCl_2$) to systems containing malt-amylase, starch, and gelatin, ovalbumin, or egg-white will be described.

Experimental.

Methods.

Solutions of Merck's starch were prepared by boiling with water. The amylase used was Merck's "Diastase absolut." Solutions not containing protein were buffered either by Sørensen's phosphate solutions or by glycine-sodium chloride-sodium hydroxide solutions. p_H was determined potentiometrically, using a calomel electrode. Gelatin purchased from Coignet Père et Fils (Paris) was used, and Merck's ovalbumin, solutions of which were prepared by dissolving in water, leaving 12 hours and filtering. The p_H of protein solutions was adjusted by addition of the requisite quantities of 0·1 *N* NaOH or HCl. The salts used were Merck's analytical reagents. The experiments were in most cases performed at 20°, and the velocity of reaction was determined either from the final reducing sugar content, determined by Bertrand's method, or from the final starch content, determined by Pflüger's glycogen method.

Results.

I. *Effect of salts in the absence of proteins.*

Action of sodium chloride. This was studied at p_H 3·6 and 5·2, *i.e.* above and below the optimal p_H 4·5. The results (Table I) show that at p_H 5·2 no effect is observed in concentrations of less than *N*; in 2*N* solutions of NaCl hydrolysis amounts to 53 % and in 3*N* solutions to 8 % of the normal value. At p_H 3·6 the inhibitory action of sodium chloride appears at lower concentrations, hydrolysis being 88·3 % in 0·1 *N*, 63 % in 0·2 *N* and 40·7 % in 0·3 *N* NaCl.

Table I. *Action of amylase in presence of NaCl.*

Systems contain 10 cc. Sørensen's phosphate buffers, 10 cc. 1·5 % starch, 10 cc. NaCl solution, and 1 cc. 0·1 % amylase. Temp. 20°. Duration of reaction: 4 hours for systems at p_H 5·2, 5 hours for systems at p_H 3·6. Maximum yield of maltose 120 mg.

	Maltose produced			
Conc. NaCl *N*	At p_H 3·6 mg.	% of yield without NaCl	At p_H 5·2 mg.	% of yield without NaCl
0	36·5	100	77·9	100
0·02	35·9	—	77·4	99·4
0·05	34·2	—	83·1	
0·10	30·2	88·3	83·6	
0·20	21·4	63·0	83·4	
0·30	13·9	40·7	83·8	107
0·60	—	—	84·4	
0·80	—	—	84·0	
1·0	—	—	83·7	
2·0	—	—	41·3	53
3·0	—	—	6·1	8
4·0	—	—	6·3	8

Action of calcium chloride. Similar results were obtained with calcium chloride, in solutions buffered to p_H 3·6, 5·2 and 6·8 (Table II). The velocity of reaction fell at p_H 3·6 to 90 % of normal in 0·072 *N* $CaCl_2$, 51·8 % in 0·144 *N*, 8 % in 0·2–0·9 *N*, and 4·2 % in 1·1 *N* salt. At p_H 5·2 the inactivating effect was observed first in 0·444 *N* solutions of $CaCl_2$, in which the velocity of hydrolysis was diminished by 18 %, and further increase in concentration of $CaCl_2$ led to a further slow decline. At p_H 6·8 three periods were observed: in the first, the addition of $CaCl_2$ enhanced hydrolysis by about 40 %, this optimal value being maintained over a range of 0·018–0·222 *N* $CaCl_2$, whilst in higher concentrations the velocity of hydrolysis again fell. Thus in 0·444 *N* solutions it amounted to 90 % of the original value, in 0·666 *N* $CaCl_2$ to 32 %, and in 0·888 *N* solutions to 1·6 %. At both p_H 5·2 and 6·8 the inhibitory action of $CaCl_2$ was first manifested in 0·444 *N* solutions.

Table II. *Effect of $CaCl_2$.*

Systems contain 15 cc. of 1·5 % starch, 1 cc. 0·1 % amylase without NaCl, and 5 cc. $CaCl_2$ or H_2O. The control systems yielded 37·7 mg. glucose on hydrolysis.

	Starch decomposed (expressed as glucose)					
Conc. $CaCl_2$ *N*	At p_H 3·6 mg.	% of yield without $CaCl_2$	At p_H 5·2 mg.	% of yield without $CaCl_2$	At p_H 6·8 mg.	% of yield without $CaCl_2$
0	16·6	100	17·7		8·6	100
0·018	17·4	106	18·2		12·7	147·6
0·086	17·9		17·7	100	12·4	144·2
0·072	14·9	90·0	18·5		12·6	146·5
0·144	8·6	51·8	17·7		12·4	144·2
0·222	1·4		17·9		13·2	153·5
0·444	1·4	8·4	14·5	82·0	11·4	132·0
0·666	2·6		8·1	46·0	4·1	47·9
0·888	1·4		4·7	26·6	0·2	1·6
1·100	0·7	4·2	4·8	26·8	0·2	

Action of aluminium chloride. The action of this salt was studied at p_H 5·2, and the solution of $AlCl_3$ was previously reduced to this p_H by addition of

0·1 *N* NaOH. The velocity of amylolysis was reduced to 65·3 % of the original value by 0·025 *N* $AlCl_3$, and to 44·4 % by 0·05 *N* aluminium chloride (Table III).

Table III. *Effect of* $AlCl_3$.

Systems contain 10 cc. 1·8 % starch, 15 cc. $AlCl_3$ or H_2O, 1 cc. 0·1 % amylase (without NaCl), and 6·7 cc. H_2O or 0·1 *N* NaOH for neutralisation of $AlCl_3$. Systems kept 4 hours at 20° p_H 5·2.

Conc. $AlCl_3$ *N*	Maltose produced mg.	% of yield without $AlCl_3$
0	34·0	100
0·025	22·2	65·3
0·050	15·1	44·4
0·100	11·6	34·1
0·200	5·1	15·0

II. *Action of salts in systems containing gelatin.*

The systems contained 0·6–1·2 % gelatin, warm solutions of which were added to starch solutions with or without electrolyte, and the mixtures were left for 12 hours, after which 1 cc. of 0·01 % amylase in 0·1 % NaCl was added with stirring to the semi-gelated mass. All systems were at p_H 5·7. Systems containing sodium chloride exhibited a feeble Tyndall effect; this was much more evident in the presence of calcium chloride.

Action of sodium chloride. The results obtained (Table IV) indicate that sodium chloride has, after a certain concentration, an activating effect, 15 % more maltose being produced in 0·02 *N*, and 23 % more in 0·04 *N* NaCl than in the absence of this salt.

In view of Freiberger's observation [1931] that the apparent reducing sugar content of solutions as determined by Bertrand's method is smaller in the presence of gelatin than in its absence, experiments were performed on solutions containing maltose and gelatin, with and without salts. The values found were the same in both cases, indicating that the activation found is not due to methodical errors, but to an actual increase in the activity of amylase.

Table IV. *Action of NaCl in systems containing gelatin.*

10 cc. 0·6 % starch, 10 cc. 1·2 % gelatin, 1 cc. 0·01 % amylase in 0·1 % NaCl, and 2 cc. of salt solution or H_2O. Systems kept 4 hours at 20° (p_H 5·7). Maximum yield of maltose 45 mg.

	Maltose produced			
	Exp. 1		Exp. 2	
Conc. NaCl *N*	mg.	% of yield without NaCl	mg.	% of yield without NaCl
0·0	9·0	100	7·3	
0·002	8·6	95·5	7·6	
0·004	8·6	95·5	7·3	100
0·008	8·7	96·7	6·9	
0·012	8·7	96·7	7·2	
0·016	9·3	103·3	7·4	
0·020	11·3	126·6	8·4	115
0·032	11·4	126·7	9·0	123·3
0·040	11·4	126·7	9·0	123·3

Action of potassium chloride. The results obtained with KCl are identical with those found for NaCl.

Action of calcium chloride. Calcium chloride at certain concentrations diminishes velocity of reaction at 18°, but not at 30°. The percentage retardation varies from 5 to 23 % in the initial stages of the reaction; given sufficient time, the reaction proceeds in all cases to completion. The addition of potassium chloride to systems containing calcium chloride diminishes the inactivating effect of the latter salt.

Table V. *Action of $CaCl_2$ in systems containing gelatin.*

10 cc. 0·6 % starch, 10 cc. 1·2 % gelatin, 1 cc. 0·01 % amylase in 0·1 % NaCl, and 2 cc. of salt solution or H_2O. Systems kept 4 hours at 20°; p_H 5·7.

		Starch decomposed		
No. of exp.	Time of reaction (hours)	Without $CaCl_2$ mg.	In presence of 0·032 *N* $CaCl_2$ mg.	% retardation
1	1	15·0	11·6	22·6
	2	40·4	35·7	11·6
2	0·5	4·4	3·8	13·6
	1	5·5	4·4	20·0
3	0·5	8·7	7·0	19·5
	1·0	15·1	12·9	14·6
	12·0	45	45	0
4	0·5	6·1	5·8	5·0
	1	11·0	11·0	9·1
	24	44·8	45·1	0

III. *Action of salts in systems containing ovalbumin.*

Amylase was added to the following systems: (*a*) 5 cc. 1 % starch solution and 5 cc. of about 4 % Merck's ovalbumin; (*b*) the same, boiled; (*c*) ovalbumin solution first boiled, and starch solution then added; (*d*) starch solution added

Table VI. *Action of dissolved and heat-coagulated ovalbumin in presence and absence of $CaCl_2$.*

The starch present was equivalent to 42·4 mg. glucose.

	Starch hydrolysed (mg.) at			
	p_H 5·2			p_H 6·1
	0·01 % amylase		0·1 % amylase	0·01 % amylase
System	1 hour	2 hours	4 hours	4 hours
(*a*) Ovalbumin + starch + amylase	11·2	19·7	35·7	8·2
(*b*) Ovalbumin + starch, heated at 100°, then amylase	12·7	20·1	35·5	8·2
(*c*) Ovalbumin heated at 100°, then starch and amylase	11·2	19·7	35·3	8·3
(*d*) Ovalbumin + starch + $CaCl_2$ + amylase	12·7	20·1	35·5	8·7
(*e*) Ovalbumin + starch + $CaCl_2$ heated at 100°, then amylase	11·2	20·1	35·5	8·3
(*f*) Ovalbumin + $CaCl_2$, heated at 100°, then starch and amylase	11·2	20·1	35·3	8·7

to ovalbumin solution containing $CaCl_2$; (*e*) the same, boiled, and (*f*) starch added to boiled ovalbumin-$CaCl_2$ solution. The experiments were carried out at p_H 5·2 and 6·06. 1 cc. of 0·2, 0·1 or 0·01 % amylase was added to all systems, and 1 cc. of water or of 1·2 *N* $CaCl_2$ solution. The results (Table VI) indicate that the velocity of hydrolysis is independent of the state of the ovalbumin (sol or gel) and of the presence of $CaCl_2$ (0·1 *N*).

IV. *Action of salts in systems containing egg-white.*

Coagulated egg-white. 1 cc. of water or $CaCl_2$ in various concentrations and 10 cc. of 1 % starch solution were added to 5 g. of heat-coagulated egg-white and the systems were shaken for 1 hour, after which 1 cc. of 0·1 % amylase in 0·1 % NaCl solution was added, and residual starch was determined after 5 hours by Pflüger's method. The results (Table VII) indicate that 0·01 *N* $CaCl_2$ accelerates hydrolysis by 5·2 %, and 0·2 *N* $CaCl_2$ by 32 %. Considerably greater activation was observed when the time of shaking before addition of amylase was prolonged to 4 hours, when the acceleration of reaction due to 0·1 *N* $CaCl_2$ was not 24 %, as after 1 hour's shaking, but 55 %, whilst in 0·2 *N* $CaCl_2$ it was now 60 %. The activating effect of the addition of calcium chloride was evident in concentrations at which no effect was observed in the absence of protein.

Table VII. *Action of $CaCl_2$ in systems containing egg-white.*

Starch present equivalent to 74·0 mg. glucose. Residual starch determined by Pflüger's method.

	Shaken 1 hour		Shaken 4 hours	
Conc. $CaCl_2$ *N*	Starch decomposed mg.	% acceleration due to $CaCl_2$	Starch decomposed mg.	% acceleration due to $CaCl_2$
0	23·2	—	27·0	—
0·01	24·4	5·2	—	—
0·02	24·6	5·3	—	—
0·05	27·3	18	34·7	28·5
0·10	28·8	24	41·5	54·0
0·20	30·6	32	44·2	64·0

The p_H of the systems was originally 8·8, but fell to 8·1 on addition of calcium chloride; this change in p_H leads to an acceleration of reaction amounting to about 43 %.

Sodium chloride (0·1 *N*) has no influence on the velocity of hydrolysis in analogous systems. It was, however, observed that the addition of NaCl to systems containing $CaCl_2$ diminished the activation due to the latter salt. Thus we see (Table VII) that in the presence of 0·1 *N* NaCl the acceleration due to 0·1 *N* $CaCl_2$ was only 23 %, whilst with 0·05 *N* $CaCl_2$ no acceleration was observed.

The addition of sodium sulphate (0·05–0·2 *N*) has no influence on the system starch-amylase-coagulated egg-white (Table VIII).

Comparison of the action of dissolved and coagulated egg-white. 5 cc. of egg-white were added to 5 cc. of water, 10 cc. of 1 % starch, 1 cc. amylase in

0·1 % NaCl, and 1 cc. of water or 2·2 *N* $CaCl_2$ were added. Egg-white was coagulated in certain of the systems by heating for 2 minutes at 100°, in the presence or absence of $CaCl_2$, and amylase was added to the cooled systems. The results (Table IX) show that in the absence of electrolytes the coagulation of egg-white reduces the velocity of reaction by 41–54 %. $CaCl_2$ in concentrations up to 0·1 *N* accelerates reaction both in the presence of dissolved and of coagulated egg-white; this acceleration amounted respectively to 40–56 % and 86–142 %, the velocity of reaction in the presence of coagulated egg-white and $CaCl_2$ being greater than in the presence of dissolved egg-white without $CaCl_2$.

Table VIII. *Systems as in Table VII, but different salts.*

Salt	Starch decomposed (expressed as mg. glucose)
—	30·4
0·1 *N* NaCl	28·9
0·1 *N* NaCl + 0·05 *N* $CaCl_2$	29·7
0·1 *N* NaCl + 0·1 *N* $CaCl_2$	37·3
—	30·8
0·05 *N* Na_2SO_4	30·8
0·10 *N* Na_2SO_4	30·2
0·20 *N* Na_2SO_4	30·2

Table IX.

Duration of reaction 4 hours; p_H without $CaCl_2$ 8·8, with $CaCl_2$ 8·1.

System	Starch decomposed mg.	% decomposition as compared with system (*a*)
(*a*) Dissolved egg-white + starch	29·2–31·5	100
(*b*) „ „ „ + $CaCl_2$	42·5–46·1	135–158
(*c*) Egg-white + starch, heated at 100°	14·1–17·0	44·8–58·6
(*d*) Egg-white + starch + $CaCl_2$, heated at 100°	31·7–34·8	109–111

Discussion.

The action of salts in systems containing amylase and starch is quantitatively different, according to whether the systems are above or below the optimal p_H of the amylase; in the former case the same effect is achieved by 10 times higher concentrations than in the latter. At p_H 7 $CaCl_2$ accelerates reaction when present in small concentrations. At p_H 5·2 the action of salts appears to be a function of the valency of the cation; thus the minimal retarding normal concentrations of the salts $AlCl_3$:$CaCl_2$:NaCl are as 1:18:60. At p_H 3·6 this dependence is not observed, the actions of the salts being roughly equal at equivalent concentrations. At p_H 5·2 and 3·6 no acceleration of reaction was observed in the presence of low concentrations of salts, and this may be ascribed to the fact that the enzyme preparation was not previously freed from salts by dialysis, and to the relative insensitiveness of vegetable amylase to mineral salts [Sherman and Tauberg, 1916; Saito, 1906]. The action of salts is very different according to the variety of protein present, and to its physical state. In systems containing gelatin, the addition of

sodium chloride leads to acceleration of reaction (23 % acceleration in 0·04 *N* solution). The causes of this effect are not clear; it may be due to combination with gelatin of the NaCl added with the amylase solution, and necessary for its action, and hence the addition of further NaCl accelerates reaction. Experiments with $CaCl_2$, however, do not support this hypothesis, but suggest that the action of NaCl is rather in the direction of affecting the degree of aggregation of gelatin, thereby facilitating the penetration of amylase into protein aggregates containing occluded starch.

Calcium chloride exerts a retarding action, in contradistinction to NaCl. Since the retarding concentrations of calcium chloride in such systems are considerably smaller than the smallest active concentrations in systems not containing protein, the explanation of its action should be connected with the presence of protein, and this view is further supported by the fact that at 30° no retardation due to the presence of $CaCl_2$ can be observed, and that, given sufficient time, the final yield of maltose is the same in the presence or absence of $CaCl_2$. Observations of the Tyndall effect and of viscosity in jellified systems indicate that $CaCl_2$ enhances the compactness of the jelly (Targońska, unpublished work), and the possibility may therefore exist that the penetration of amylase within starch-containing aggregates is rendered more difficult in the presence of this salt.

The results of experiments conducted on systems containing ovalbumin or egg-white are even more difficult of interpretation than are those for gelatin. Filipowicz (unpublished work) has found that the velocity of reaction of amylase with starch is accelerated by the addition of proteins, in systems having p_H values considerably higher than the optimal for amylase; this acceleration is considerable, and may under suitable conditions amount to over 40 %. A second factor influencing the velocity of reaction is the change in p_H due to addition of neutral salts; this, as has been shown by Przyłęcki and Giedroyć [1931], may be quite considerable in alkaline systems in the presence of protein. Filipowicz has, however, found that changes in p_H have a much feebler effect on the activity of amylase in systems containing protein than in its absence. In this case, we see (Table X) that 0·1 *N* $CaCl_2$ reduced the p_H of a system containing protein and starch from 8·8 to 8·1, and that this change in p_H itself leads to an acceleration of reaction of 43·3 %.

Table X.

Systems contain 5 cc. egg-white, 5 cc. water, 10 cc. 1 % starch, 1 cc. 0·1 % amylase, and 1 cc. of $CaCl_2$, HCl, or water.

System	p_H	Starch decomposed mg.	% acceleration
As above, with water	8·8	33·3	—
,, HCl	8·1	47·7	43·3
,, $CaCl_2$	8·1	50·0	50·1

As to possible changes produced by salts on the state of combination (this we shall term "sorption," without entering into the question of the chemical,

physical, or mechanical nature of the forces to which the phenomenon is due) of starch or amylase with protein, our knowledge is very inadequate, at least in so far as systems containing protein sols are concerned. Both NaCl and $CaCl_2$ diminish sorption, to a greater extent when added before coagulation of the sol than when added after. The reversal of the process of sorption, where it has already taken place, requires considerably higher concentrations of sodium chloride than of calcium chloride, and the process of desorption proceeds very slowly in both cases, equilibrium being attained in about 4 hours. In systems to which $CaCl_2$ has been added after coagulation of protein 64 % of the total starch remains in solution after shaking for 1 hour, whilst in the absence of $CaCl_2$ only 55 % remains, a difference of 17 %. After a further 3 hours, the corresponding figures are 51 and 27 %, and the difference now amounts to 51 %. When $CaCl_2$ (0·1 *N*) is added before coagulation of protein, 55 % of the total starch remains in solution, as compared with 20 % in the absence of $CaCl_2$, or 175 % more. Sodium chloride, in the concentrations taken (up to 0·05 *N*), had very little influence on the proportion of starch remaining in solution (22 % with NaCl, 15 % without).

Coagulated ovalbumin combines with starch to a much smaller extent than does coagulated egg-white. Neither dissolved nor coagulated ovalbumin retards the velocity of amylolysis, either in the presence or absence of $CaCl_2$, whence it appears that in such systems the reduction in concentration of the substrate in solution is without effect on the velocity of reaction, in spite of the fact that in homogeneous systems the latter is reduced by about 50 % by a similar change in concentration. The velocity of reaction is, further, independent of whether the ovalbumin was coagulated in the presence or the absence of starch. Occluded starch is liberated in alkaline solution in the same way as sorbed starch, in contradistinction to acid systems (p_H 3), in which both occlusion and sorption are irreversible [Przyłęcki and Gurfinkel, 1930; Przyłęcki and Majmin, unpublished work].

In systems containing egg-white quite different results were obtained, the velocity of reaction being considerably smaller in the presence of coagulated than of soluble protein. This relative retardation amounts in the systems studied to about 50 %, whilst at the same time sorption amounts to about 80 %. The disproportionality between these figures may be ascribed to the partial combination of starch or enzyme with protein sol; alternative explanations would be that it is due to the greater viscosity of systems containing soluble proteins, or that the reason for the smaller velocity observed in the presence of coagulated egg-white lies not in sorption but in change in concentration of activating protein. The effect of addition of $CaCl_2$, however, supports the view that sorption is the cause of retardation of reaction, as this salt accelerates reaction in the presence of dissolved and coagulated egg-white respectively by 50 and above 100 %.

This activation may be due largely to reduction in p_H of unboiled systems, in which a change from p_H 8·8 to 8·1 accelerates reaction by 43 %; this effect

does not, however, apply equally to systems containing coagulated egg-white. Here the following facts support the view that $CaCl_2$ acts by desorption, with consequent increase in active concentration. The same concentration of $CaCl_2$ has more than twice as great an effect in systems containing coagulated than dissolved egg-white. The percentage acceleration of reaction is more than doubled by shaking systems containing coagulated egg-white and $CaCl_2$ for 4 hours instead of 1 hour, whilst where egg-white is coagulated in the presence of $CaCl_2$ it is about four times as great. These changes in the velocity of reaction vary roughly in parallel with the variations in the proportion of dissolved to sorbed starch in the above systems. In systems containing soluble egg-white, $CaCl_2$ may, apart from its usual effect of lowering the p_H of the systems, diminish the proportion of starch combined with protein particles. No obvious explanation is forthcoming as to the reason why the velocity of reaction in the presence of coagulated egg-white and $CaCl_2$ is greater than in systems containing dissolved egg-white without $CaCl_2$. It is possible that certain of the effects observed are due to variations in the active concentration of enzyme due to its adsorption on dissolved or coagulated protein; work on this aspect of the question is now being conducted.

The above observations may possess certain biological applications, chiefly in plant physiology. It is evident that vegetable cells possess agents, such as $CaCl_2$, which are able to affect the quantitative relation between free and sorbed starch in the cytoplasm, and that such influences may determine the velocity of enzymic hydrolysis; in systems in which the reaction of synthesis is in equilibrium with that of hydrolysis the presence of mineral salts may even determine the direction of reaction.

Summary.

1. Electrolytes retard the velocity of enzymic hydrolysis of starch. Systems of p_H below the optimal are more sensitive to the action of salts than are those of p_H higher than the optimal; in the latter case the active concentration of ions is a function of their valency.

2. The velocity of enzymic hydrolysis in systems containing gelatin is accelerated by the addition of NaCl, and retarded by the addition of $CaCl_2$.

3. In systems containing Merck's ovalbumin neither heat-coagulation nor $CaCl_2$ affects the velocity of reaction.

4. In systems containing crude egg-white heat-coagulation in the presence of $CaCl_2$ accelerates reaction. The velocity depends on the time during which the system containing coagulated egg-white and $CaCl_2$ is shaken before addition of amylase. NaCl and Na_2SO_4 are without effect in this system. In the absence of $CaCl_2$ heat-coagulation reduces velocity of reaction by 50 %.

5. The velocity of reaction in the presence of dissolved egg-white is increased by about 50 % by addition of $CaCl_2$. This effect is due to reduction

of p_H, as the same acceleration is obtained by addition of the appropriate quantity of HCl.

6. In systems containing heat-coagulated egg-white and $CaCl_2$, the acceleration observed is due probably to desorption of starch from protein.

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CLXX. THE OXIDATION OF SULPHYDRYL COMPOUNDS BY HYDROGEN PEROXIDE.

I. CATALYSIS OF OXIDATION OF CYSTEINE AND GLUTATHIONE BY IRON AND COPPER.

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The oxidation of thiol compounds by oxygen and methylene blue has, in recent years, been very extensively studied, while their oxidation by hydrogen peroxide has suffered almost complete neglect. Abderhalden and Wertheimer [1923] found that cysteine, like other mercaptans, was instantaneously oxidised by hydrogen peroxide in neutral solution, and that this oxidation was unaffected by cyanide. At neutrality an oxidation of the thiol group would be complicated, at any rate in the presence of iron, by the Fenton reaction. Quastel, Stewart and Tunnicliffe [1923] have indeed prepared an oxidation product of this type from glutathione. In acid solution and in the presence of the theoretical amount of hydrogen peroxide the reaction proceeds smoothly with the production of pure oxidised glutathione [Pirie, 1931].

In this paper the results of a study of the oxidation of cysteine and glutathione to the corresponding disulphides will be given while in a later one a more deep-seated oxidation will be described. The former action is catalysed by iron, copper and thiocarbamide while the latter is catalysed by vanadium, titanium and molybdenum. The following elements have, in acid solution, either no action or a very slight one: Ag, As, Be, Co, Cr, Hg, Mn, Ni, Pb, U, W, Zn.

Experimental.

The course of the oxidation was followed polarimetrically since the ordinary methods which have been used for following oxidations are clearly inapplicable. Polarimeter tubes were made of heavy-walled glass tubing, 4 mm. internal diameter and 20 cm. long, with a side-tube through which they were filled. The ends (end plates for the ordinary type of polarimeter tube) were fixed on with de Khotinsky cement. Except for the part over the bore of the tube the end plates were blackened. When not in use the tubes, and all the other vessels used in this work, were kept filled with 20 % hydrochloric acid. Before use they were rinsed out five or six times with glass-distilled water and dried by connecting to a mechanical pump having an efficient sulphuric acid absorption tube.

"Metal-free" cysteine hydrochloride was prepared according to Dixon's method by recrystallisation four times from metal-free 20 % hydrochloric acid; metal-free glutathione was obtained by the method already described [Pirie, 1930] the vessels used for washing and decomposing the cuprous compound being washed with acid before use. Merck's "perhydrol" has a very low metal content and was used in all cases diluted to give a 0·44 *N* solution. At this concentration it is stable for some days but if diluted much further it tends to decompose.

Glass apparatus was used throughout, and, presumably because all the fluids used were acid, no trouble was experienced through metals being adsorbed by the glass.

The oxidation of cysteine.

In the absence of added metal the rate of oxidation of cysteine is very small at p_H 2·1 (the p_H of a dilute solution of cysteine hydrochloride) but is markedly increased by small amounts of iron or copper. These results are shown in Fig. 1. In each case the appropriate amount of a dilute solution of ferrous sulphate (freshly prepared each day) or copper sulphate was added to 1 cc. of a 1 % solution of cysteine hydrochloride, the amount of metal-free water necessary to make up the volume to 4·5 cc. was run in from a burette and finally 0·5 cc. of 0·44 *N* H_2O_2 was added. The mixture was then poured into a dry polarimeter tube and the rotation measured for mercury green light. It is clear that in each case the rate of oxidation is proportional to the amount of metal added over a fairly wide range. The temperature coefficient of the reaction was calculated from the velocities at 10° and at 20° in the presence of 0·0013 mg. Cu and of 0·002 mg. Fe and was found to be 2·0 (± 0·1) in the first case and 2·2 in the second.

The catalytic effects of iron and copper are sharply differentiated from each other by the different effects of variations in the concentrations of cysteine and H_2O_2. The rate of oxidation in the presence of copper is proportional to the peroxide concentration and independent of the amount of cysteine, whereas in the presence of iron it depends on cysteine and is independent of peroxide. (Curves illustrating this phenomenon in the case of iron are given later when the effect of phosphate is considered.) This effect is somewhat different from that studied by Elliott [1930] in the aerobic oxidation of cysteine; in this case the rate of oxidation depended on the cysteine concentration for both iron and copper catalysis. Elliott also found that, with 8 mg. of cysteine hydrochloride and at p_H 7·3, 0·0042 mg. Cu caused maximum rate of oxidation. It is apparent from Fig. 1 that there is no such limit in the case of oxidations at a lower p_H.

Effect of p_H. The effect of an increase in p_H on the catalytic oxidation is complicated by the fact that the rate of oxidation in the absence of added metal is also greatly increased and, at neutrality, is much too rapid to be followed conveniently by this technique. I have worked mainly at p_H 4·6.

1 cc. of 1 % cysteine hydrochloride was conveniently buffered at this p_H by 0·3 cc. of $M/3$ metal-free ammonium acetate, which was prepared by neutralising redistilled acetic acid with distilled ammonia. It is clear from Fig. 2 that this change in p_H has affected the rate of oxidation in the absence of added metal or in the presence of iron to a greater extent than that in the presence of copper. This suggests that the unavoidable oxidation is due to the presence of traces of iron. It is not of course permissible to compare these rates of change of rotation directly, for the specific rotations of cystine and cysteine vary with p_H. Cysteine in acid solution has a small positive rotation whereas

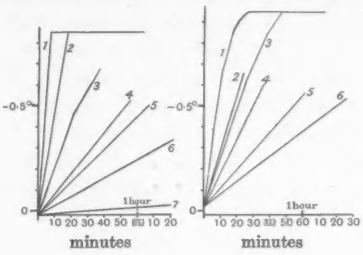

Fig. 1.

Fig. 2.

Fig. 1. Volume made up to 5 cc. in each case. Temperature 20°. p_H 2·1.

1.	1 cc. 1 % cysteine-HCl;	0·02 mg. Cu;	0·5 cc. 0·44 *N* H_2O_2.
2.	,,	0·05 mg. Fe;	,,
3.	,,	0·003 mg. Cu;	,,
4.	,,	0·001 mg. Fe;	,,
5.	,,	0·001 mg. Cu;	,,
6.	,,	0·0005 mg. Fe;	,,
7.	,,	No metal	,,

Fig. 2. Volume made up to 5 cc. in each case. Temperature 20°. p_H 4·6.

1.	1 cc. 1 % cysteine-HCl;	0·001 mg. Fe;	0·3 cc. $M/3$ ammon. acetate;	0·5 cc. 0·44 *N* H_2O_2.
2	,,	0·0003 mg. Fe;	,,	,,
3.	,,	0·001 mg. Cu;	,,	,,
4.	,,	No metal;	,,	2·0 cc. 0·44 *N* H_2O_2.

5. 2 cc. cysteine-HCl; 0·6 cc. ammon. acetate; 1 cc. H_2O_2 incinerated *etc.* then 1 cc. cysteine-HCl; 0·3 cc. ammon. acetate and 0·5 cc. H_2O_2.
6. 1 cc. 1 % cysteine-HCl; 0·3 cc. $M/3$ ammon. acetate; 0·5 cc. 0·44 *N* H_2O_2.

Bergmann and Michalis [1930] found that cysteine itself was slightly laevorotatory in aqueous solution. I find that this shift occurs at about p_H 4, and it is shown by a 0·04° change in the initial rotations in Figs. 1 and 2. Cystine undergoes a change in rotation at about the same p_H. Andrews [1925] found that the specific rotation of cystine, dissolved in hydrochloric acid, increased with the dilution. Lutz and Jirgensens [1930] showed that, with the same concentration of cystine, the rotation increased as the concentration of the acid or alkali used to dissolve it diminished. On account of its insolubility the rotation of cystine was not measured by these workers at neutrality. Supersaturated solutions of cystine are however relatively stable and, as Fig. 2 shows, the rotation of a very highly supersaturated solution of cystine does not alter for at least 40 minutes. By this method the specific rotation

of cystine for mercury green light and in 0·2 % solution has been determined at varying p_H.

p_H	$[\alpha]^{20}_{5461}$	p_H	$[\alpha]^{20}_{5461}$
2·1	$-276° \pm 2$	5·5	-312
2·5	-279	7·7	-297
3·1	-286	8·3	-227
4·6	-312	9·0	-156

In a later section when the effects of various inhibitors are considered the reason for the fairly high rate of oxidation in the absence of added metal will be discussed more fully. It has already been shown that the effect of p_H on the rate suggests that it is a case of iron catalysis. Curve 4, Fig. 2, shows however that at least the greater part of it is not due to iron, since a fourfold increase in H_2O_2 concentration increases the rate threefold, whereas control experiments show that at p_H 4·6, as at p_H 2·1, the rate of oxidation in the presence of iron is independent of the concentration of H_2O_2.

A similar conclusion was reached by testing the reagents separately for catalytic power after incineration. The following method was found to be the most reliable. A silica test-tube ($10 \times 1{\cdot}5$ cm.) was heated with a very small oxy-coal gas flame at a point a little to one side of the bottom and then drawn out with a piece of silica rod to form a horn 2 mm. long and 1·5 mm. broad at the base. The tube was clamped in a sloping position with the horn downwards and heated with a micro-burner giving a flame about 1·5 mm. broad. If care were taken that only the tip of the horn was touched by the flame the incineration proceeded smoothly without any bumping. Sulphuric acid and ammonia were freed from metal by distillation although ordinary pure ammonia (sp. gr. 0·880) is generally sufficiently free from metals already. The results of these experiments are somewhat inconclusive on account of the danger of contamination during the fairly lengthy incineration which is necessary with such a low metal concentration. 0·1 cc. of concentrated sulphuric acid was used and one drop of strong ammonia solution (sp. gr. 0·93) was added before the mixture was heated, this reduced the time necessary for complete incineration to about 15 minutes. The mixture was cooled, and 2 cc. of water were added followed by slight excess of ammonia. The excess ammonia was removed by cautiously boiling till ammonium sulphate crystallised. 1 cc. of cysteine hydrochloride solution was now added and, after a minute or two, 0·3 cc. of $M/3$ ammonium acetate, 0·5 cc. of 0·44 N H_2O_2 and the volume made up to 5 cc. Curve 5 on Fig. 2 is the result of incinerating 2 cc. of cysteine hydrochloride, 1 cc. of H_2O_2 and 0·6 cc. of ammonium acetate in this way. Similar experiments on the reagents separately showed that the greater part of this metal came from the cysteine hydrochloride. Ammonium sulphate at this concentration is without effect on the rate of oxidation either in the presence or the absence of added metal, though higher concentrations, as for example when 0·6 cc. of sulphuric acid is used for the incineration, inhibit the oxidation in both cases.

At p_H 1·6 the rate of oxidation in the presence of copper is only slightly less than at p_H 2·1, whereas the oxidation due to iron is greatly diminished. At this p_H cystine is fairly soluble, the effect of added cystine was therefore determined. Elliott [1930] has shown that, at p_H 7·3, cystine catalyses the copper but not the iron catalysis of cysteine oxidation. It is apparent from Fig. 3 that it catalyses neither type of oxidation appreciably at p_H 1·6. Metal-free cystine hydrochloride was made by precipitation with hydrogen chloride as described by Gerwe [1931]. A 1 % solution of this in N/9 HCl was used. In plotting the rotations measured in the presence of cystine, 0·86°, the rotation due to the cystine, has been subtracted in each case. Curves

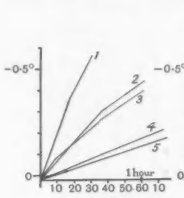

Fig. 3.

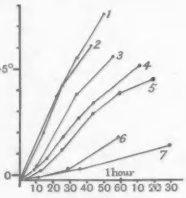

Fig. 4.

Fig. 3. Volume made up to 5 cc. in each case. Temperature 22°. p_H 1·6.

1. 1 cc. 1 % cysteine-HCl; 1 cc. N/9 HCl; 0·01 mg. Fe; 0·5 cc. 0·44 N H_2O_2.
2. ,, 1 cc. N/9 HCl; 0·001 mg. Cu; 0·5 cc. 0·44 N H_2O_2.
3. ,, 1 cc. 1 % cystine-HCl in N/9 HCl; 0·001 mg. Cu; 0·5 cc. 0·44 N H_2O_2.
4. ,, 1 cc. 1 % cystine-HCl in N/9 HCl; 0·001 mg. Fe; 0·5 cc. 0·44 N H_2O_2.
5. ,, 1 cc. N/9 HCl; 0·001 mg. Fe; 0·5 cc. 0·44 N H_2O_2.

Fig. 4. Volume made up to 5 cc. in each case. Temperature 19°. p_H 2·1.

1. 2 cc. 1 % cysteine-HCl; 0·002 mg. Fe 0·88 mg. KH_2PO_4; 0·5 cc. 0·44 N H_2O_2.
2. 1 cc. 1 % cysteine-HCl; 0·002 mg. Fe; no phosphate; ,,
3. ,, ,, 0·44 mg. KH_2PO_4; ,,
4. ,, ,, 0·88 mg. KH_2PO_4; ,,
5. ,, ,, ,, 1·0 cc. 0·44 N H_2O_2.
6. ,, 0·001 mg. Fe; 0·44 mg. KH_2PO_4; 0·5 cc. 0·44 N H_2O_2.
7. ,, 0·002 mg. Fe; 2·2 mg. KH_2PO_4; ,,

showing the effect of cystine on iron catalysis in the presence of phosphate are given later.

Effect of phosphate. Copper catalysis is unaffected by even M/100 phosphate both at p_H 2·1 and 4·6. Iron catalysis on the other hand is strongly inhibited at both reactions. This is shown in Fig. 4. The stock phosphate solution was M/62 KH_2PO_4, and even 0·2 cc. *i.e.* M/1550, was strongly inhibiting. As curves 1, 4 and 5 show, the rate of oxidation in the presence of iron, even when it is partially inhibited by phosphate, depends on the cysteine concentration and is independent of the concentration of H_2O_2. The slight inhibition shown in curve 5 has been obtained several times and becomes large if a large amount of H_2O_2 is added. In the presence of 0·00064 M phosphate the rate of oxidation, during the first hour at any rate, is no longer proportional to the iron present. In the presence of phosphate, therefore, cysteine behaves like thioglycollic acid [Elliott, 1930] so far as iron catalysis is con-

cerned. The situation is, however, complicated by the autocatalytic shape of the curves. Dixon and Tunnicliffe [1922] observed this autocatalysis in the methylene blue oxidation of .SH groups at p_H 7·6 but not at p_H 6. Fig. 5 shows that it is not due to catalysis by the cystine formed for, in the presence of iron and at p_H 1·6, the oxidation is autocatalytic when phosphate is present but is not catalysed by cystine. The autocatalysis does not seem to be due to a difference between ferric and ferrous phosphate for the curves are the same whether the iron and cysteine are first mixed together and allowed to stand for 10 minutes before the addition of the phosphate and peroxide, or the iron and peroxide mixed first and then the other reagents added. It is possible, though from a quantitative point of view unlikely, that the increase in the

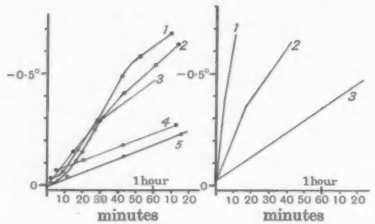

Fig. 5.

Fig. 6.

Fig. 5. Volume made up to 5 cc. in each case. Temperature 22°.

1. 1 cc. 1 % cysteine-HCl; 1 cc. 1 % cystine-HCl in $N/9$ HCl; 0·01 mg. Fe; 0·66 mg. KH_2PO_4; 0·5 cc. 0·44 N H_2O_2.
2. 1 cc. 1 % cysteine-HCl; 1 cc. $N/9$ HCl; 0·01 mg. Fe; 0·66 mg. KH_2PO_4; 0·5 cc. 0·44 N H_2O_2.
3. 1 cc. 1 % cysteine-HCl; 6 mg. sodium pyrophosphate; 0·62 mg. Fe; 0·001 mg. Cu; 0·5 cc. 0·44 N H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 6 mg. sodium pyrophosphate; 0·62 mg. Fe; 0·001 mg. Cu; 0·5 cc. 0·44 N H_2O_2. The reagents being added in this order.
5. 6 mg. sodium pyrophosphate; 0·62 mg. Fe; 0·5 cc. 0·44 N H_2O_2; 1 cc. 1 % cysteine-HCl. The reagents being added in this order.

Fig. 6. Volume made up to 5 cc. in each case. Temperature 22°. p_H 4·6.

1. 1 cc. 1 % cysteine-HCl; 0·3 cc. $M/3$ ammon. acetate; 0·0005 mg. Fe; 0·5 cc. 0·44 N H_2O_2.
2. " ; " ; 1·6 mg. KH_2PO_4; " "
3. " ; " ; 2·2 mg. KH_2PO_4; no metal; " "

rate of oxidation as the oxidation proceeds is due to the disappearance of H_2O_2 since the latter exerts a slight inhibitory effect. If this were the explanation, the initial rate of oxidation of a mixture, made up as in curve 4, Fig. 4, but with only 0·4 cc. of H_2O_2, should be the same as the rate after 20 minutes with 0·5 cc. Actually the two curves are identical.

At p_H 4·6 the inhibitory effect of phosphate is, as might be expected from the work of others, less marked. A pair of curves illustrating this is given in Fig. 6 and also a control curve in the presence of 0·0032 M phosphate. A comparison of this curve with the similar one in Fig. 2 shows that the inhibition is hardly outside the experimental error; this suggests, in agreement with the incineration results, that only a small part of the unavoidable oxidation can be due to iron.

Effect of pyrophosphate. Copper catalysis is unaffected by pyrophosphate unless the latter is present in very high concentration. Catalysis by iron on the other hand is inhibited quantitatively by pyrophosphate even when there is present only slightly more than one molecule of pyrophosphate for each molecule of iron. When there is a large amount of iron present (0·02 mg.) it can be inhibited by less than one molecule of pyrophosphate to one of iron, but a precipitate forms after a few minutes and makes the fluid in the tube opaque. No precipitate is formed in the presence of excess pyrophosphate. Fig. 7 shows the effect of pyrophosphate on iron catalysis at p_H 4·6, and it is clear

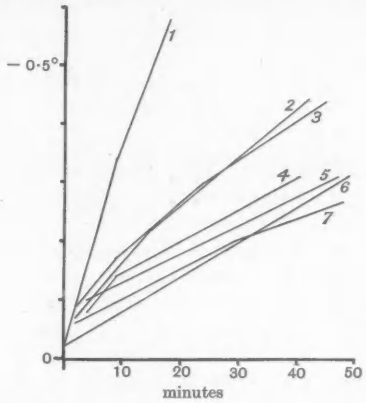

Fig. 7. Volume made up to 5 cc. in each case. Temperature 20°. p_H 4·6.

1. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·001 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2; 0·0032 mg. $Na_4P_2O_7.10\,H_2O$.
2. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·001 mg. Fe; 0·008 mg. pyrophosphate; 0·5 cc. H_2O_2.
3. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·062 mg. Fe; 0·0002 mg. Cu; 0·8 mg. pyrophosphate; 0·5 cc. H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·001 mg. Fe; 0·011 mg. pyrophosphate; 0·5 cc. H_2O_2.
5. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·001 mg. Fe; 0·016 mg. pyrophosphate; 0·5 cc. H_2O_2.
6. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; no metal or pyrophosphate; 0·5 cc. H_2O_2.
7. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·062 mg. Fe; 0·8 mg. pyrophosphate; 0·5 cc. H_2O_2.

that copper may be detected by this technique in the presence of 300 times its weight of iron. The curves for iron in the presence of a substantial excess of pyrophosphate show that the latter has only a very slight effect on the "blank." Experiments with pyrophosphate but without added iron show that not more than 15 % of the unavoidable oxidation can be due to iron. The curves in Fig. 7 show that there is always some rapid oxidation at the beginning of each experiment. Two curves are given in Fig. 5 which indicate that this is due to a difference between ferric and ferrous pyrophosphate since it is not obtained when the cysteine is added last. Fig. 5 also shows that, in

the presence of 620 times its weight of iron, copper is somewhat inhibited by pyrophosphate.

Effect of HCN. Metal-free HCN was prepared by distilling a mixture of sodium cyanide and sulphuric acid and collecting the distillate in water. The results are summarised in Fig. 8. By comparison with Fig. 1 it is apparent that cyanide activates iron at p_H 2·1 while it has a hardly appreciable inhibitory action on copper. At p_H 4·6 on the other hand it inhibits small amounts of copper completely and has a slight inhibitory effect on iron. The difference between curve 2 on Fig. 2 and curve 1 on Fig. 8 is however not

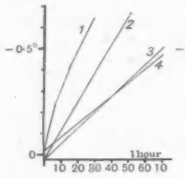

Fig. 8.

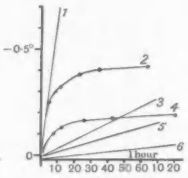

Fig. 9.

Fig. 8. Volume made up to 5 cc. in each case. Temperature 22°.

1. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; 0·0003 mg. Fe; 0·27 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
2. 1 cc. 1 % cysteine-HCl; 0·001 mg. Fe; 0·54 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
3. 1 cc. 1 % cysteine-HCl; 0·001 mg. Cu; 1·0 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 0·3 *M*/3 ammon. acetate; 0·003 mg. Cu; 0·54 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; no metal; 0·54 mg. HCN; 0·5 cc. 0·44 *N* H_2O_2.
4. 1 cc. 1 % cysteine-HCl; 0·3 cc. *M*/3 ammon. acetate; no metal; no HCN; 0·5 cc. 0·44 *N* H_2O_2.

Fig. 9. Volume made up to 5 cc. in each case. Temperature 20°. p_H 2·1.

1. 1 cc. 1 % cysteine-HCl; 0·002 mg. Fe; 0·4 mg. ferrocyanide; 0·5 cc. 0·44 *N* H_2O_2.
2. ,, ,, 1·0 mg. ,, ,,
3. ,, no metal; 1·0 mg. ,, ,,
4. ,, 0·001 mg. Cu; 1·0 mg. ,, ,,
5. ,, ,, 0·2 mg. ferricyanide; ,,
6. ,, ,, 1·0 mg. ,, ,,

marked. A curve is also given in Fig. 8 showing that HCN has no effect on the rate of oxidation in the absence of added metal.

Effect of ferro- and ferri-cyanide. No attempt has been made to analyse fully the somewhat intricate systems to which these reagents give rise. They are slightly catalytically active themselves in the absence of added metal and, since the rate of oxidation seems to increase as the cyanide becomes more dilute, this is probably due to the decomposition of the cyanide. Fig. 9 shows that ferrocyanide activates iron at first and that this effect later gives place to an almost complete inhibition. Ferricyanide gives no such activation. Ferricyanide in the same way seems to inactivate copper almost completely at p_H 2·1 while ferrocyanide has first to be oxidised to ferricyanide (*cf.* curves 4 and 6 in Fig. 9). At p_H 4·6 the catalytic effect of these compounds masks any inhibitory effect which they may exert.

A number of other substances has been tested, using concentrations of about 1 mg. in 5 cc., as inhibitors for copper or iron. Carbon monoxide, urea, urethane, glutamine, alanine and histidine have no effect on copper; glutamic acid and potassium thiocyanate do not affect iron. The various reagents[1] introduced by Eichholtz [1929] and Krah [1930] were without effect on either metal when used in the maximum concentration possible with this technique. Owing to the colour of the reagents and to their insolubility in acid solution the concentration was generally 0·1 mg./cc. Sulphuretted hydrogen was converted immediately to free sulphur by the H_2O_2 present.

It was at first thought that low results which were often got with iron after incineration might be due to inhibition by pyrosulphate. The addition of pyrosulphate, however, inhibits neither iron nor copper at p_H 2·1 or 4·6. For some reason, therefore, the method of incineration used is not satisfactory if one wishes to estimate iron although it works perfectly in the case of copper.

Catalysis by copper derived from haemocyanin.

It is well known, from the work of Roche [1930] and others, that haemocyanin is not stable in acid solution. Haemocyanins from three species, *Helix pomatia*, *Limulus polyphemus* and *Maia squinado*, have been examined and in each case the copper appears to be completely liberated in catalytically active form at p_H 2·1. This, in the case of *Helix* is shown in Fig. 10, where the rates of oxidation in the presence of serum and of the same amount of serum after incineration are compared. This comparison has been made repeatedly and there has never been any difference between serum or dialysed serum and incinerated serum so far as catalytic activity is concerned. In only one sample of serum was there any detectable difference between the rates of oxidation in the presence and in the absence of pyrophosphate, the iron content of the sera of these three animals is therefore less than 1/20 of the copper content.

At p_H 4·6 neither *Helix* nor *Limulus* haemocyanin has any catalytic activity at all. In curve 6, Fig. 10, the crosses give the rate of oxidation in the absence of haemocyanin and the line gives two determinations of the rate in the presence of some snail haemocyanin. Curve 2 shows the effect of adding the same amount of haemocyanin to the cysteine hydrochloride before the ammonium acetate instead of after it. It makes no difference if the acetate is added 10 minutes after the haemocyanin instead of 20 seconds after, *i.e.* the decomposition at p_H 2·1 is complete in less than 20 seconds.

Maia haemocyanin is less stable than the other two and has a slight catalytic activity at p_H 4·6. Curves 3, 4 and 5 in Fig. 10 illustrate the decomposition which it undergoes in 3 minutes at p_H 3·9 and 3·65.

Since it is perfectly stable at p_H 3·3 this was the p_H used for the oxidation with *Helix* haemocyanin, the decomposition resulting from 3 minutes at

[1] 8-Hydroxyquinolinesulphonic acid, 1-amino-8-naphthol-4-sulphonic acid, 1-amino-2-naphthol-6-sulphonic acid, 5-nitro-4-chloro-2-aminophenol.

p_H 2·4, 2·7 and 2·9 is shown in Fig. 11. In these and all other cases the amount of ammonium acetate necessary to give any required p_H was found from a titration curve. $M/3$ ammonium acetate was run into 0·2 % cysteine hydrochloride solution and the p_H measured with a hydrogen electrode. Curves 1 and 2 on Fig. 11 show that, as might be expected, the rate of oxida-

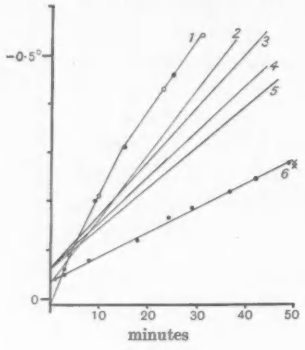

Fig. 10.

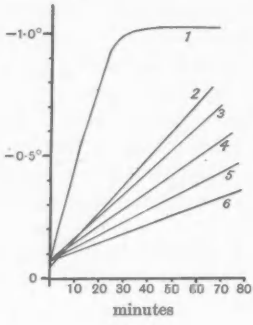

Fig. 11.

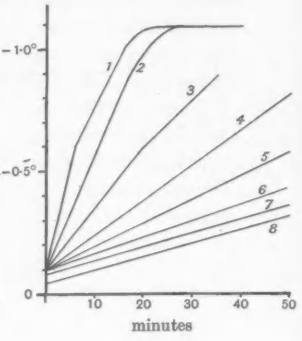

Fig. 12.

Fig. 10. Volume made up to 5 cc. in each case. Temperature 21°.

1. —.—.— 0·047 cc. of *Helix pomatia* serum; 1 cc. 1 % cysteine-HCl; 0·5 cc. 0·44 *N* H_2O_2.
   —o—o— 0·047 cc. of *Helix pomatia* serum incinerated; 1 cc. 1 % cysteine-HCl; 0·5 cc. 0·4 *N* H_2O_2.
2. 1 cc. 1 % cysteine-HCl; 0·01 cc. *Helix* serum; 0·3 cc. $M/3$ ammon. acetate; 0·5 cc. 0·44 *N* H_2O_2.
3. 4. 5. 0·03 cc. of *Maia* serum left for 3 minutes with a mixture of 1 cc. cysteine-HCl and acetate at the p_H stated. The rest of the acetate and the H_2O_2 were then added — 3: p_H 3·65; 4: p_H 3·9; 5: p_H 4·6
6. 1 cc. 1 % cysteine-HCl; 0·3 cc. $M/3$ acetate; 0·01 cc. *Helix* serum; 0·5 cc. 0·44 *N* H_2O_2.

× × Extent of oxidation of controls after 50 minutes.

Fig. 11. Volume made up to 5 cc. in each case. Temperature 20°.

1. 1 cc. 1 % cysteine-HCl; 0·067 cc. *Helix* serum; 0·5 cc. 0·44 *N* H_2O_2.
2. " 0·017 cc. *Helix* serum; "
3. 4. 5. 6. 0·017 cc. *Helix* serum left for 3 minutes with a mixture of 1 cc. cysteine-HCl and acetate at the p_H stated. The rest of the acetate, and the 0·5 cc. of 0·44 *N* H_2O_2 were then added. — 3: p_H 2·4; 4: p_H 2·7; 5: p_H 2·9; 6: p_H 3·3
6. Control at p_H 3·3 with no haemocyanin.

Fig. 12. Temperature 20°. In each case the volume is 5 cc. and the final p_H is 4·6. An amount of *Limulus polyphemus* serum containing 0·0032 mg. of copper was added to each after the addition of the amount of ammonium acetate solution necessary to bring 1 cc. of 1 % cysteine-HCl to the required p_H. After a suitable time the rest of the ammonium acetate was added and then 0·5 cc. of 0·44 *N* H_2O_2.

1. 3 minutes at p_H 2·1.
2. 3 minutes at p_H 2·9.
3. 10 minutes at p_H 3·3.
4. 3 minutes at p_H 3·3.
5. 20 seconds at p_H 3·3.
6. 3 minutes at p_H 3·75.
7. 3 minutes at p_H 4·6.
8. Control at p_H 4·6.

tion at p_H 2·1 is proportional to the amount of haemocyanin. The effect of variations in the concentration of haemocyanin on the extent of its decomposition when the decomposition is only partial has not been measured.

In Fig. 12 curves are given illustrating the effect of p_H on the stability of *Limulus* haemocyanin. In 3 minutes a barely perceptible decomposition takes

place at p_H 3·75, but at lower p_H the decomposition is rapid. Three curves at p_H 3·3 show the effect of different times of exposure to acid. In all these experiments the actual oxidation takes place at p_H 4·6. Figs. 11 and 12 both show that when the oxidation is allowed to proceed to completion in the presence of decomposed haemocyanin the final rotation observed is more than 0·1° higher than that of the cystine formed. This extra rotation is due to the degraded haemocyanin. Natural haemocyanin has a small negative rotation which is doubled or trebled on the addition of acid, the addition of a corresponding amount of alkali does not reduce the rotation again to its old level. For example:

Rotation of 2 dm. of a solution of dialysed *Helix* haemocyanin containing 0·005 mg. of copper per cc.	$= -0{\cdot}08°$
Rotation after the addition of 0·2 cc. of N H_2SO_4 to 10 cc. of haemocyanin. p_H now 1·8	$= -0{\cdot}18°$
Rotation after adding 1 cc. of $N/5$ NaOH to this	$= -0{\cdot}16°$

This last value was the mean of several readings made by two observers, since on neutralising a solution of haemocyanin which has been acid a very considerable turbidity develops. This was observed in all the experiments in which the stability of haemocyanin at varying p_H was measured, but, if the decomposition were not carried more than half-way, it is fairly slight.

Catalysis by haematin and haemoglobin.

The catalysis which is observed with these two substances is due, in part at any rate, to inorganic iron liberated under the conditions of the experiment. A few minutes' contact with H_2O_2 in the presence of cysteine hydrochloride is sufficient to discharge the colour of haemoglobin and, after this treatment, haematin will no longer give a haemochromogen with pyridine; both, on the other hand, now give an iron test with potassium thiocyanate. This decomposition takes place at both p_H 2·1 and 4·6.

The oxidation of glutathione.

In the presence of copper the oxidation of glutathione is very similar to that of cysteine. The rate of oxidation depends on the concentrations of copper and of hydrogen peroxide but it is independent of the concentration of glutathione. Thus curves 3 and 4 in Fig. 13 have the same gradient although the initial rotations are different since one represents only half as great a concentration of glutathione as the other. This rate of oxidation is doubled in curve 2, which has twice the H_2O_2 concentration, and in curve 1, with 0·006 instead of 0·002 mg. of copper, it is trebled. Meldrum and Dixon [1930] found that, for the aerobic oxidation of glutathione, small quantities of a third factor were necessary. Mason [1931] suggested that this was cysteinylglycine. This factor is of only secondary importance in the copper-catalysed oxidation by hydrogen peroxide. The sample of glutathione used in this work was very

pure, having been recrystallised from aqueous solution at about the freezing point. It gave theoretical analytical figures. A few cc. of the 2 % solution used were heated at 100° in a steam-bath for 35 minutes and, after cooling, used for oxidation experiments. The rate of oxidation in the presence of 0·006 mg. of copper or in the absence of added metal at p_H 4·6 was unaltered by this treatment although a considerable amount of decomposition is known to occur.

When the oxidation of glutathione is catalysed by iron it differs markedly from the oxidation of cysteine. The rate of oxidation is no longer proportional to the metal concentration but decreases suddenly when there is less than 1 part of iron for each 2000 of glutathione. Elliott [1930] found a very similar state of affairs in the iron-catalysed aerobic oxidation of thioglycollic acid.

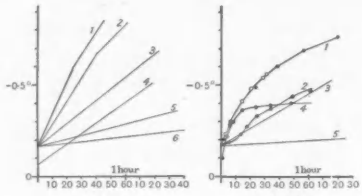

Fig. 13.

Fig. 14.

Fig. 13. Volume made up to 5 cc. in each case. Temperature 21°.

1. 1 cc. 2 % reduced glutathione; 0·006 mg. Cu; 0·5 cc. 0·44 *N* H_2O_2.
2. 1 cc. 2 % reduced glutathione; 0·002 mg. Cu; 1·0 cc. 0·44 *N* H_2O_2.
3. 1 cc. 2 % reduced glutathione; 0·002 mg. Cu; 0·5 cc. 0·44 *N* H_2O_2.
4. 0·5 cc. 2 % reduced glutathione; 0·002 mg. Cu; 0·5 cc. 0·44 *N* H_2O_2.
5. 1 cc. 2 % reduced glutathione; no metal; 0·3 cc. *M*/3 ammon. acetate; 0·5 cc. 0·44 *N* H_2O_2.
6. 1 cc. 2 % reduced glutathione; no metal; 0·5 cc. 0·44 *N* H_2O_2.

Fig. 14. Volume made up to 5 cc. in each case. Temperature 21°.

1. —.—.—.— 1 cc. 2 % reduced glutathione; 0·01 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2.
   —o—o—o— 1 cc. 2 % reduced glutathione; 0·01 mg. Fe; 1·0 cc. 0·44 *N* H_2O_2.
2. 1 cc. 2 % reduced glutathione; 0·01 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2; 0·18 mg. KH_2PO_4.
3. 1 cc. 2 % reduced glutathione; 0·008 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2.
4. 0·5 cc. 2 % reduced glutathione; 0·01 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2.
5. 1 cc. 2 % reduced glutathione; 0·005 mg. Fe; 0·5 cc. 0·44 *N* H_2O_2.

As with cysteine the rate of oxidation is independent of the peroxide concentration but is, as would be expected, an inverse function of the glutathione concentration. These facts are illustrated in Fig. 14.

A preliminary heating of the glutathione to 100° seems to increase the rate of oxidation in the presence of iron. The effect is small however and, since any decomposition will result in a lowering of the concentration of glutathione and this has already been shown to cause an increased rate of oxidation, it may well be unconnected with the production of cysteinylglycine.

Inhibitors, so far as they have been studied, have a similar effect on both oxidations. Cyanide inhibits copper partially at p_H 2·8 while phosphate is without effect. Phosphate inhibits iron strongly and at certain concentrations (*M*/4000) the curve is slightly inflected (curve 2, Fig. 14).

Discussion.

The nature of the oxidation occurring at p_H 4·6 cannot be considered to be definitely settled but it seems very probable that all the oxidation at this p_H is not due to traces of metals. It behaves in a manner unlike iron catalysis so far as variations in H_2O_2 concentration are concerned and it is not inhibited by phosphate or pyrophosphate. It behaves on the other hand unlike copper catalysis towards variations in p_H and it is not inhibited by cyanide. Furthermore, if an experiment similar to that given in curve 5 of Fig. 2 is carried out, but with the addition of 0·1 mg. of pyrophosphate, the rate of oxidation is identical with that of the "blank" in the presence of pyrophosphate. This indicates that what metal there is, is iron. In a later paper I hope to describe the catalysis of this reaction by thiocarbamide. This oxidation will proceed rapidly in the absence of added metal and the effect of metal is, over a certain range at any rate, simply additive. The existence of this latter example of a "metal-free" oxidation by hydrogen peroxide lends colour to the idea that the present oxidation also can proceed in the absence of metal. At Prof. Haldane's suggestion the temperature coefficients of the copper- and iron-catalysed oxidations and of the oxidation without added metal were measured. In each case the coefficient was $2 \pm 0{\cdot}2$. No distinction between the three types of oxidation can therefore be drawn in this way.

At p_H 6 the oxidation of cysteine, without the addition of metal, is complete in a few minutes, the final rotation being 0·95° as in the case of the metal-catalysed oxidation. The final rotation when the oxidation occurs at p_H 4·6 cannot be determined since the cystine begins to crystallise after about 2 hours, *i.e.* before the reaction has gone to completion. Even in the presence of a very substantial excess of peroxide (1 cc. of 0·064 *M* cysteine hydrochloride or 1 cc. of 0·066 *M* glutathione mixed with 0·5 cc. of 0·44 *N* H_2O_2) the rotation of an acid solution of either cystine or oxidised glutathione will remain constant for over 12 hours, while the expected amount of hydrogen peroxide remains in the solution and can be estimated by titration. In acid solution therefore, in the presence of iron and copper and in the case of glutathione of the decomposition products formed by boiling for a short time, hydrogen peroxide can only react very slowly, if at all, to bring about further oxidation. On the alkaline side further decomposition readily takes place. In this connection the specific rotation of oxidised glutathione has been measured at several different reactions. Mercury green light was used and the solution, initially 1 %, was diluted somewhat at the higher p_H since in all 1·5 cc. of *N*/5 sodium hydroxide were added to 6 cc. of glutathione solution.

p_H	2·85	$[\alpha]^{21}_{5461}$	$-115° \pm 1$	p_H 7·0	$[\alpha]^{21}_{5461}$ -133
	5·0		-125	8·6	-150
	6·0		-126	9·6	-176

Glutathione resembles cysteine in showing a slight increase in specific rotation as the p_H increases from about 3 to 5. Unlike cysteine, however, it shows a further increase as the p_H rises above 5.

It is obvious that this catalytic reaction offers a very convenient method for estimating small amounts of copper, if necessary in the presence of iron. The sensitivity is about the same as that of Warburg's method [1927] but the technique is easier and, since all the solutions are acid, vessels simply need to be rinsed out 3–4 times with glass-distilled water after use. With the polarimeter available in this laboratory readings are accurate to 0·01° (in general the observed points have only been put on those curves which are non-linear; if the points on the graphs had been given a diameter corresponding with this probable error they would all have touched the lines which have been drawn) quantities of copper of the order of 0·001 mg. may therefore be estimated with an error of about 5 %. Metals such as vanadium will of course interfere with this method of estimation.

Summary.

In the presence of copper the rate of oxidation of cysteine by hydrogen peroxide at p_H 2·1 is proportional to the concentrations of copper and peroxide but independent of that of cysteine.

In the presence of iron it is proportional to the concentrations of iron and cysteine and is slightly inhibited by an increase in H_2O_2.

At p_H 4·6 there is some metal-free oxidation and the rate of oxidation in the presence of iron is much increased while that in the presence of copper is less markedly affected.

Figures are given for the specific rotations of cysteine and glutathione between p_H 2·1 and 9·0.

Phosphate inhibits iron and gives an inflected oxidation curve when the inhibition is incomplete. Pyrophosphate inhibits iron stoichiometrically.

At p_H 2·1 cyanide activates iron and hardly affects copper, at p_H 4·6 both are inhibited. Ferricyanide inhibits copper and iron, but ferrocyanide activates iron and only inhibits copper when it has been oxidised.

Haemocyanin has no catalytic effect but at p_H 2·1 it is decomposed completely giving catalytically active copper. The stability of three different haemocyanins at varying p_H has been measured.

Haematin and haemoglobin are not stable under the conditions used.

The rate of oxidation of glutathione in the presence of copper is proportional to the concentrations of copper and H_2O_2 and independent of that of glutathione. In the presence of iron there is a sort of auto-inhibition and the rate of oxidation is an inverse function of the glutathione concentration; it is independent of the H_2O_2.

This reaction is suggested as a convenient method for estimating small amounts of copper.

I am deeply indebted to Mrs K. G. Terroux for the various samples of haemocyanin and to Sir F. G. Hopkins for his interest and advice.

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CLXXI. THE ABSORPTION OF WATER BY GELATIN.

VI. THE INFLUENCE OF THE THICKNESS AND ORIGINAL CONCENTRATION OF THE GEL.

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Introduction.

In the previous papers in this series on the behaviour of dry leaf gelatin when immersed in watery media, the effects of variations in the composition of the external medium have been studied [Jordan-Lloyd, 1920, 1930; Jordan-Lloyd and Pleass, 1927, 1928; Pleass, 1929, 1930]. It is, however, obvious that if (as frequently postulated in the course of the work) gelatin gels are systems with a definite internal architecture, variations in the methods used in preparing the dry gelatin might also be expected to influence the behaviour of the system. It has indeed been recognised from the earliest days of investigations into the physical chemistry of gelatin that the characteristics of the particular sample of gelatin used must be taken into consideration. Hofmeister [1890] and Pauli a few years later [1897] both introduced a factor allowing for the thickness of the dry gelatin in equations expressing the rate of swelling of gelatin in water. The idea that both external and internal dimensions of the dried gelatin play a part in its subsequent behaviour in water has indeed been latent in much of the literature, the ratio of surface to mass, for instance, having been considered by other workers besides Hofmeister and Pauli. The problem of the influence of the internal structure of the gelatin has, however, only been investigated in comparatively recent times.

Procter [1911] showed that the swelling of dried gelatin gels in water was partly controlled by the percentage concentration at which the original gelatin jelly had been allowed to set. Using gelatin dried from 5, 10 and 20 % jellies, he showed that swelling in water was inversely proportional to the original concentration. Gortner and Hoffmann [1922] confirmed this observation; using gelatin gels dried from jellies of 2·5, 5, 10, 15 and 20 % concentration, they showed that both the rate and amount of swelling in water were inversely proportional to the concentration at setting. Their experimental figures also give an indication that in contact with water the 10 % gel has a stability of concentration greater than that of gels which are more concen-

trated or more dilute, for, while gels dried down from more concentrated jellies regain more water on soaking than they lost during evaporation, and gels dried down from less concentrated jellies do not regain as much water as they lost, the gel dried from a 10 % jelly returns approximately to its former state on soaking in water. The special stability of the 10 % gelatin jelly has been demonstrated in a very attractive manner by Kunitz [1928], who used jellies of different gelatin concentrations from 3 to 20 % and immersed them without previous drying in $M/1000$ acetate buffer solutions at p_H 4·7. All the jellies which contained more than 10 % of gelatin absorbed water and all those which contained less gave up water to the surrounding fluid. Northrop [1927] and Northrop and Kunitz [1927] had previously shown that the equilibrium weight of gelatin jellies in water could be expressed by an equation involving the concentration at setting as a variable, and Kunitz, in his later paper, showed that the equation applied equally whether that equilibrium were attained by either gaining or losing water.

The work reported in the present paper deals with the influence of both thickness and original concentration of the jelly on the swelling of gelatin gels in water and in salt solutions, and at acid and alkaline reactions. The experimental evidence shows that both the thickness of the gelatin pieces and the original concentration of the gels at setting influence the amount of water absorbed at equilibrium by gels under different external conditions. This is equally true whether the gels are placed in the experimental conditions in the undried state or whether they have been taken to dryness as a first stage. The experiments, however, also show that the process of drying the gels at temperatures below their melting points very greatly reduces the capacity to absorb water under all the experimental conditions studied (the influence of the drying being inversely proportional to the original concentration) and that, therefore, changes occur in the gelatin gels during drying, which are not fully reversed on restoring to an aqueous medium.

Method and materials.

Most of the experiments were carried out with Coignet's Gold Label Gelatin, purified by the method previously described [Jordan-Lloyd and Pleass, 1927], but as large quantities of material were required, the commercial gelatin was sometimes used without purification. The latter possesses the practical advantage over the purified gelatin of being sufficiently soluble to make a 50 % gel. The gelatin used is, in every case, indicated in the experimental records. The gelatin was dissolved by soaking in cold water and then warming to 50° and adjusting the volume to the required concentration. Gels were cast by pouring the warm sol into moulds of hard paraffin wax and, when required for use in the dry condition, were dried as rapidly as possible at room temperature under an electric fan.

The experiments were carried out by placing the weighed gelatin pieces in stoppered glass jars with the experimental solution and a small amount

of toluene to prevent bacterial growth. Water absorption was followed in every experiment by weighing at suitable intervals of time until equilibrium (or substantial equilibrium) had been attained. Time curves are not given in this paper, but equilibrium may be considered to be reached at 23° in 3 days, at 18° in 4 days, at 7° in 7 days, at 0° in 10 days. Gels of pure gelatin come to a true equilibrium at 0° and 7°, but continue to absorb water slowly for an indefinite period at higher temperatures, due possibly to the solubility of the gelatin at these temperatures or else to the development of "fatigue" [see Northrop, 1927]. Gels of commercial gelatin do not come into a true equilibrium at any temperature, though at low temperatures they come to a state in which further changes are slow. In most cases, the absorption of water from (1) distilled water, (2) 0·2 *M* sodium chloride solution, (3) *N*/1000 hydrochloric acid, (4) *N*/1000 sodium hydroxide was followed at various temperatures. In some cases, experiments on absorption of water from salt solutions were extended to include a range of concentration from 0·1 to 2·0 *M* and also to include sodium nitrate and sulphate as well as chloride.

Experiments on the thickness of the gelatin.

Experiments on the influence of the original thickness of the experimental pieces were carried out, both on undried and on dried gels. For comparative purposes, the thickness of the undried gels was not measured in the undried condition but was measured from the unused portion of the experimental castings after drying. Gels of 5, 10 and 15 % original concentration were used in the experiments on thickness and a range of from 0·06 to 0·65 mm. of dry thickness was covered. Thin sheets of gelatin cannot be cast from the 15 % gels since the surface forces prevent the warm sol from spreading over the wax moulds unless a minimum thickness can be attained. Experimental results at 0° both for dried and undried gels in water, salt, acid and alkaline solutions are shown in Fig. 1 (*a*) and (*b*). It can be seen that with water and sodium chloride solution the effect of the thickness is comparatively slight, although in all cases the thin pieces contain a higher percentage of water than the thick ones. With the acid and alkaline solutions, however, the thickness of the pieces has a very considerable influence on the amount of water absorbed. In Fig. 1 (*c*) these swelling curves for undried 5 % gels have been plotted against the logarithm of the thickness and it can be seen that the points lie on two straight lines. The same relation holds for the 10 and 15 % gels. Hence the relation of swelling to the thickness of the pieces can be expressed by the equation:

$$S = -k_1 \log d + k_2,$$

where S is the swollen weight at equilibrium, d is the dry thickness and k_1 and k_2 are constants. The values of k_1 and k_2 depend partly on the original concentration of the gel and partly on such factors as p_H and temperature. For any particular value of the initial concentration, it seems highly probable

that $k_2 = f(k_1)$, under conditions including both acid and alkaline swelling. There are not, however, sufficient experimental data to establish this definitely.

Further, experiments on the influence of thickness on water absorption of gels after drying bring out two points clearly: firstly, that under similar experimental conditions very much less water is absorbed than by undried

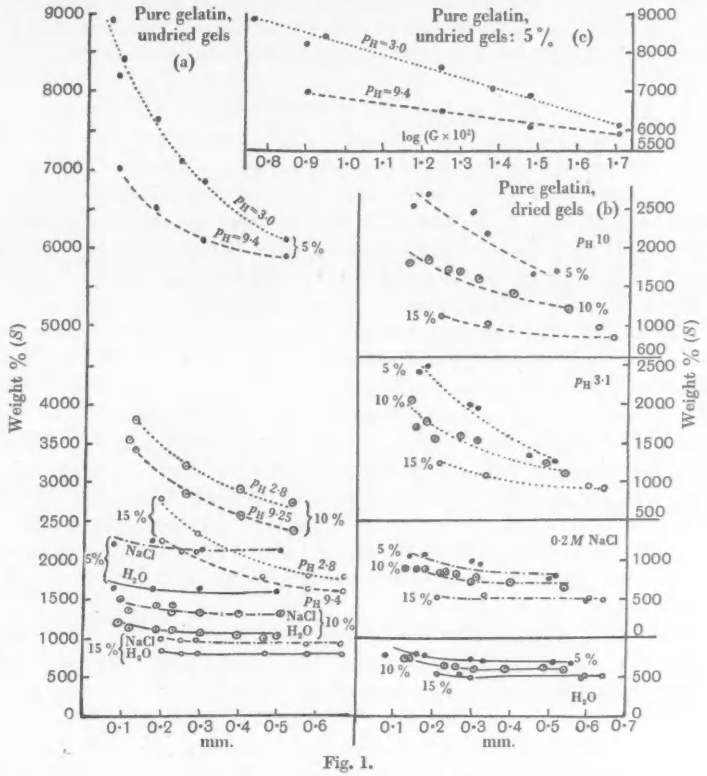

Fig. 1.

gels; secondly, that the influence of thickness of the gels has become less in the case of the acid and alkaline systems. The equation connecting swelling and thickness of the gel in water and salt solutions over the range of thickness covered by these experiments approximates to the form

$$S = -k_3 d + k_4.$$

The curve for swelling in acid and alkaline solutions also approximates to the linear form, though obviously the value for k_3 is considerably greater than in the case of water and salt solutions.

The effect of drying the gel is a little difficult to explain, but it is clear that the lower the original concentration of the gel, the greater the effect of the drying. It, therefore, seems possible that during drying there is a reorganisation of the molecules of gelatin and of water within the gel of such a kind that the dried gel corresponds in internal structure to a more concentrated system than the jelly from which it has been dried. A re-arrangement of this type would lead to a reduction in the amount of water that would be absorbed on re-soaking. Details of the relation between swelling and the concentration of gelatin in the jelly are dealt with in the next section.

The effect of the initial concentration of the gel.

Experiments were made both with the original freshly set jellies and with gels which had been brought to the dry condition. The water absorption of both types was studied under various conditions. Experiments with pure gelatin were made at 5, 10, 15 and 20 % original concentration; experiments with commercial gelatin were made at 5, 10, 20 and 50 % original concentration. Observations were made at 0°, 7°, 18° and 23°. The factor for thickness was controlled, 0·25–0·30 mm. being usually chosen.

The freshly set jellies in water at 0° reach a steady equilibrium in the course of a few days, which remains steady up to 3 weeks, beyond which their behaviour was not followed. The 5 % gel lost weight; the 10 % changed very little, generally, however, showing a slight gain; the 15 and 20 % gels both gained in weight (see Figs. 1 (*a*) and 2 (*a*)). At all other temperatures all the gels gained in weight. In 0·2 *M* sodium chloride solution at 0° the gels also reach a steady equilibrium. Under these conditions the 5 % gel shows very little change of weight; the other gels all gain. At higher temperatures all gain. In dilute hydrochloric acid and sodium hydroxide solutions all gels absorb water freely at 0° and 7° and pass into solution at 18° and 23° after a few days.

The weight gained under various conditions, plotted against the original concentration, is shown in Fig. 2 (*a*). Under all circumstances, the total amount of water in the gel when it is in substantial equilibrium with its surroundings is an inverse logarithmic function of the original concentration. In Fig. 2 (*b*) the same experimental data shown in Fig. 2 (*a*) have been plotted against $1/\log G$ as abscissa, where G is the original concentration of the gelatin in the jelly. It can be seen from both figures that all the curves of swelling against concentration form a single family of curves in spite of variations of temperature and of the nature and concentration of the electrolyte in the system. The influence of initial concentration on swelling can, therefore, be expressed by the general equation:

$$S = k_5\left(\frac{1}{\log G}\right) + k_6,$$

where S is the swelling, G the original concentration and $k_6 = f(k_5)$.

Swelling, therefore, is uniformly modified under all conditions by the

properties of the gel. These properties remain fundamentally the same under different physical and chemical conditions and are, therefore, to be attributed to the mechanical properties of the gel. Without any great straining of probabilities, the influence of concentration on swelling may be attributed to the mechanical resistance of the tangled micellae in the gel to being moved, either further apart or nearer together. If the effect of concentration on swelling under any particular conditions be considered, it follows from relations of the curves to each other that the resistance to swelling diminishes not only with a fall in the initial concentration, but also under all conditions which

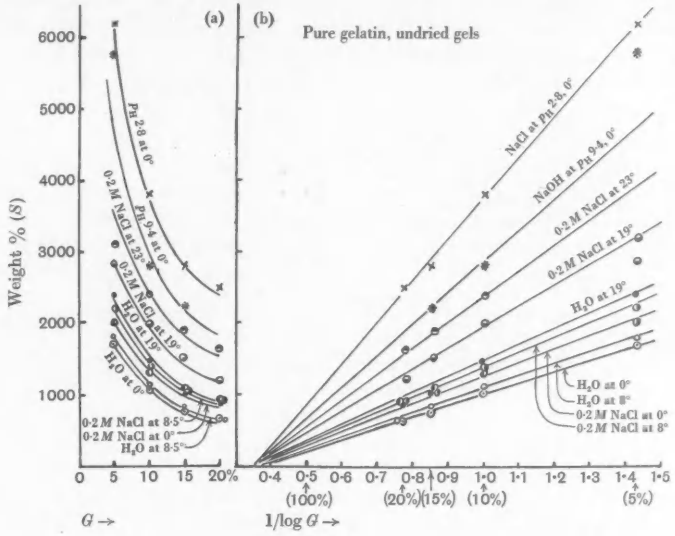

Fig. 2.

lead to an increase in the amount of water present in the gel, or in other words, the ease with which the micellae are pushed apart increases with the distance between them.

The influence of the original concentration of the gelatin in dried gels is shown in Fig. 3 (*a*) and (*b*). Curves for swelling plotted against $1/\log G$ are given both for pure gelatin (Fig. 3 (*a*)) and for commercial gelatin (Fig. 3 (*b*)). As in the experiments on thickness, the most striking effect of drying the gels before putting them under the different experimental conditions is that under all circumstances the amount of water absorbed into the system is very considerably reduced, especially in the weaker gels. As long as the original concentration of the gelatin is not greater than 20 %, the general effect of concentration on swelling can still be expressed by the general equation:

$$S = k_5\left(\frac{1}{\log G}\right) + f(k_5),$$

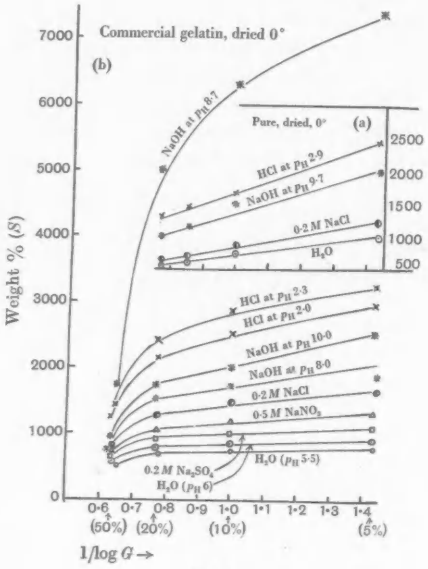

Fig. 3.

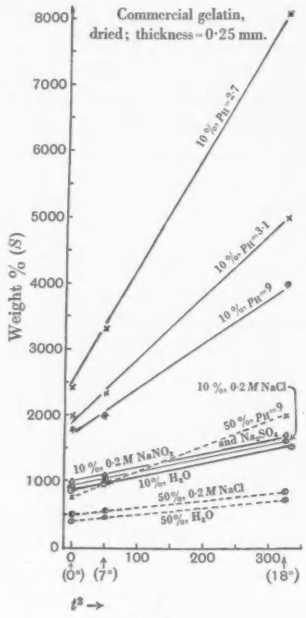
Commercial gelatin, dried; thickness = 0·25 mm.
10 %, P_H 2·7
10 %, P_H 3·1
10 %, P_H 9
10 %, 0·2 M NaCl
50 %, P_H 9 and Na_2SO_4
10 %, 0·2 M $NaNO_3$
10 %, H_2O
50 %, 0·2 M NaCl
50 %, H_2O
Weight % (S)
(0°) (7°) (18°)
t^2 →

Fig. 4

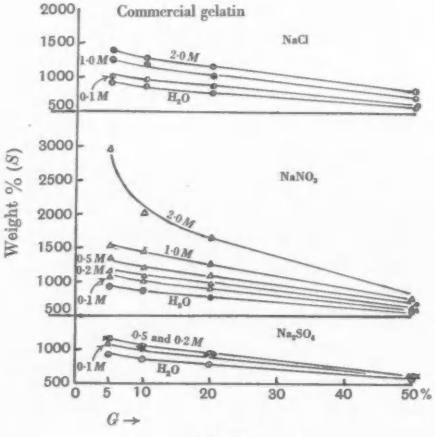

Fig. 5.

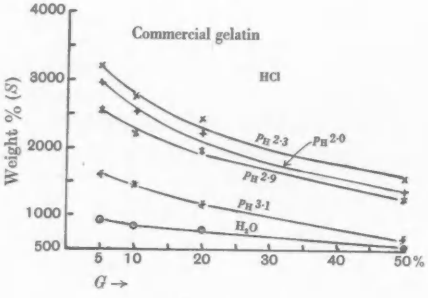

Fig. 6.

but the values of the constants k_5 and $f(k_5)$ have become different. The points obtained for the 50 % (commercial) gels do not, however, lie on the curve calculated from the general equation. It seems highly likely that both in undried jellies and dried gels this equation does not hold if the original concentration of the gelatin rises above about 20 %.

The curves for the swelling of the commercial gelatin cover a wide range of external conditions. The swelling of the gelatin has been followed not only for sodium chloride, but also for sodium nitrate and sodium sulphate. Further experimental data for these three salts over a range of concentrations up to $2M$ are given in Fig. 5. It can be seen that the influence of concentration on the water absorption remains similar with all three salts over the range of concentration studied. The values of the constants in the general equation will, however, be affected by the nature and concentration of the salt. Considering each salt individually, it is again found that the curves of swelling for different concentrations of salt form a family of curves which, if extended, come to a point at a value of $S = 100$, $G = 100$; *i.e.* under conditions where the gel contains no water, the presence of salt would fail to cause swelling. The same relation holds for the series of curves of swelling in hydrochloric acid at different values of p_H (Fig. 6).

The effect of temperature.

The effect of temperature on water absorption by gels of different thicknesses and original concentration have been studied both for the undried jelly and the dried gelatin. Experimental data are given in Fig. 7. For all systems studied, *i.e.* for water, salt, acid and alkaline solutions, the curves of weight plotted against temperature are true parabolas over a temperature range of 0°–18°, *i.e.* $S \propto t^2$, where t is the temperature in degrees centigrade. The curves for the pure gelatin gels (undried and dried) are not shown with S plotted against t^2, but Fig. 4 shows the data for commercial gelatin under a number of experimental conditions plotted on this system. The resulting curves are linear, hence under all conditions

$$S = k_7 t^2 + k_8,$$

where t is the temperature (centigrade) within the limits 0° to 18°. Where the experimental curves depart from the general parabolic form as the temperature rises, the gelatin has begun to dissolve in the surrounding medium. The relative positions of the curves (Fig. 7) suggest that the constant k_8 is influenced by both the thickness and the original concentration of the gel, but these two factors do not influence the value of the partial differential dS/dt, except possibly when the gels were set either at a low percentage of gelatin or in a very thin sheet. (In this connection, it will be noticed, on comparing the data given here with those published in the previous papers of the series, that a parabolic curve was not previously obtained for variations of swelling with temperature in water (p_H 5·0) and very dilute alkalis (p_H 7·0).

The sheet gelatin used in the previous work was obtained by dialysing commercial gelatin and was very thin, the average thickness of the pieces being 0·06 mm. compared with the 0·20–0·30 mm. used in the present work. The relatively greater surface exposed to solvent action is probably sufficient to account for the difference in experimental data.)

The value of the constant k_7 is evidently influenced by the nature of the electrolytes in the system, being very much greater in acid or alkaline than in water or salt solutions (Figs. 4 and 7). The value of the constant k_7 does not appear to be influenced by drying the gel, while the value of k_8 is considerably affected. The suggestion already put forward in this paper that a re-organisation occurs in the gel during drying in such a way as to bring

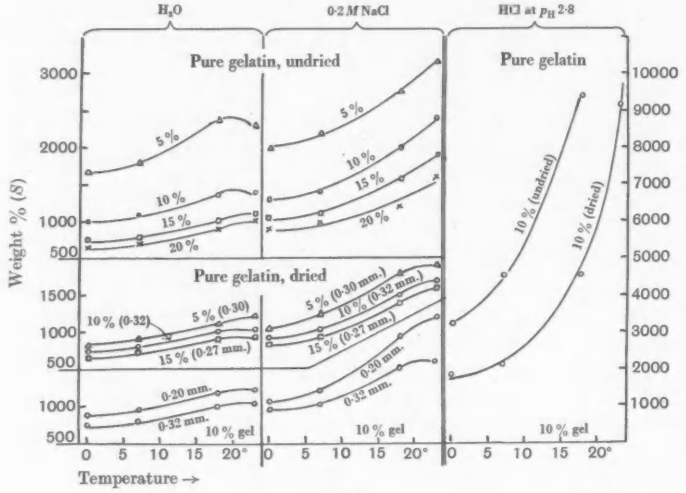

Fig. 7.

about an approximation in structure to one which set at a higher concentration of gelatin, receives support from the influence of drying on the value of k_8, which, it has already been shown, is influenced by the original concentration.

The influence of temperature on swelling is, however, so similar in nature under all external conditions that it seems clear that the predominating effect of the temperature is on some factor common to all the systems; this factor might be the gelatin itself or it might be the water. It has already been shown by Smith [1919] that gelatin in solution in water undergoes a reversible mutarotation with change of temperature. Below 15°, the "gel" form, gelatin B with $[\alpha]_D = 141°$, is the only stable form; at temperatures above 35°, the "sol" form, gelatin A with $[\alpha]_D = -313°$, is the only stable form. This transformation of the gelatin with change of temperature might be considered

responsible to some extent for lowering of the cohesive forces of the gelatin which occurs with rise of temperature, but from Smith's experimental figures only 3 % of gelatin B will be present at 18°, while from the results recorded in this paper, as soon as the concentration of gelatin B rises above this figure, solution of the gelatin sets in and equation $S = k_7 t^2 + k_8$ no longer holds. The mutarotation of gelatin with temperature, therefore, is not a factor in this equation. The influence of the temperature on the activity of the water is, therefore, probably to be regarded as the direct cause of the influence of temperature on swelling. The fact that $S \propto f(t^2)$ on the centigrade scale, where the zero is based on the property of water, is noteworthy in this connection.

In acid and alkaline solutions temperature has such a profound influence over the whole range that there seems little doubt that it influences not only the activity of the water but also the osmotic and other properties of the gelatin salts formed under these conditions.

The special stability of the 10 % gel.

It has already been mentioned in the introduction to this paper that under certain conditions 10 % gels are more stable than those of any other concentration. This observation, originally recorded in the literature by Kunitz for experiments made at 5°, is fully confirmed by the experiments recorded here. The stability of the gelatin jellies in a system free from electrolytes is not independent of either the temperature or the purity of the gelatin. It has been observed constantly throughout this work that commercial gelatin never comes into true equilibrium with its surroundings, but after nearly attaining equilibrium continues to absorb water and gain in weight for an indefinite period. The lower the temperature, the more nearly is a true equilibrium reached, and an approximate equilibrium may be taken as a measure of the change in weight due to imbibition. With pure gelatin, a true equilibrium is reached at low temperatures (0° and 7°) but not at higher ones (18° and 23°), a situation possibly to be attributed partly to the appearance of Smith's soluble B form of gelatin at the higher temperatures. The rate at which the end of the true swelling of the gelatin is reached varies with the temperature, but the following times were found by experiment to represent the end of the first rapid water absorption: 0° in 10 days, 7° in 7 days, 18° in 4 days, 23° in 3 days.

Table I shows the weights attained in equilibrium in water by gels which had been set over a range of concentration from 5 to 50 %. Figures are given both for undried jellies and gels after drying.

It will be seen here again that, at the low temperatures, all gels tend to move towards the final concentration of 10 % or 1000 parts by weight of swollen gelatin to every 100 parts of gelatin in the system. At the higher temperatures, where imbibition is undoubtedly followed by slow solution, all gels tend to draw water into the system.

Table I.

Concentration of jelly at setting ...	5 %	10 %	15 %	20 %	50 %
Original weight of jelly for 100 g. gelatin (g.)	2000	1000	670	500	200
	Weight of gel after soaking in water for 100 g. gelatin (g.)				
Pure gelatin jellies (undried) (dry thickness 0·24–0·28 mm.)					
10 days at 0°	1700	1050	760	550	—
7 „ 7°	1800	1070	800	570	—
4 „ 18°	2300	1350	830	810	—
3 „ 23°	∞	∞	1070	1000	—
Pure gelatin gels (dried before soaking) (thickness 0·27–0·32 mm.)					
10 days at 0°	1100	750	700	—	—
7 „ 7°	1150	800	770	—	—
4 „ 18°	1100	970	950	—	—
3 „ 23°	1200	1050	1000	—	—
Commercial gelatin gels (dried before soaking) (thickness 0·22–0·25 mm.)					
10 days at 0°	920	850	—	750	450
7 „ 7°	1020	950	—	800	470
4 „ 18°	1750	1500	—	1250	750
3 „ 23°	2000	1700	—	1600	900

The special stability of the 10 % gel is shown most clearly in systems which have not been dried. After drying, the re-organisation which has occurred has partly masked the effect of the original concentration. The special stability of the 10 % gel is not apparent in systems where electrolytes are present. The presence of acid, alkali or salt alters the equilibrium between the gelatin and the water. There is some evidence, however, that the tendency for gels of originally higher or lower concentration is to move in their final equilibrium towards the final equilibrium of the 10 % gel.

Discussion.

The fact that gelatin jellies absorb water and swell in aqueous solutions until they have reached an equilibrium weight has always been recognised as evidence of cohesive forces due to the gelatin itself [Procter, 1914]. These cohesive forces have also always been regarded as of a mechanical nature. It has been shown by the experiments recorded in the present paper that the total cohesive force of any gel is influenced both by its ratio of surface to mass (thickness) and by the original concentration of the jelly at setting. With dried gelatin the cohesion has been further raised by the fact of drying.

A fact which emerges clearly from a review of the experimental data given in this paper is that under most circumstances the resistance of any gel to swelling is directly proportional to the amount of gelatin present in unit volume. (An apparent exception to this generalisation is found in freshly set jellies containing less than 10 % of gelatin, which eliminate water, *i.e.* undergo actual shrinkage. Equilibrium in water differs, however, from equilibrium in solutions of electrolytes, since neither osmotic forces nor hydration due to

salt action are factors in the final equilibrium.) It is obvious, therefore, that there is some definite structure in the gel which is imposing a restraint on swelling, and that this force of inhibition becomes increasingly greater, the greater the concentration of gelatin at the moment of setting. It is not unreasonable to suppose that this force acting against the swelling forces is due to a tangle of long threadlike micellae formed in the gel, and the purely mechanical nature of the cohesive forces is confirmed by the fact that thin sheets of gelatin offer less resistance to swelling than thick ones.

The absorption of water by gelatin is, therefore, affected, not only by the properties of the solution, but also by those of the gelatin gels. Three independent factors are to be attributed to the gels themselves: (1) a tendency for all gels in the presence of water to come to an equilibrium at 10 % concentration of gelatin; (2) an elastic resistance to swelling, which is purely mechanical and can be attributed to the forces required to move the entangled, threadlike micellae apart, and which falls off as the interstitial spaces between the micellae increase in volume; (3) in dry gelatin only, an additional effect which is to be attributed to some re-organisation occurring during drying.

Summary.

1. The absorption of water under varying conditions by discs of gelatin is influenced by the thickness of the disc. The swelling of both dried and undried gelatin gels in water or salt solutions is not greatly influenced by the thickness of the gel; in acid or alkaline solutions swelling is a negative function of the logarithm of the thickness.

2. The absorption of water by gelatin is, under all conditions, an inverse logarithmic function of the original concentration at which the jelly was set. This is most clearly shown in the undried jellies.

3. The amount of water absorbed by gelatin from water, solutions of salts, acids or alkalis is a function of the square of the temperature (degrees centigrade), as long as solution of the gel itself is inappreciable.

4. The influence of the original concentration of the gel depends on the amount of water in the system, irrespective of whether the water has penetrated into the jelly through the direct action of temperature, p_H value of the solution or the nature of the salts present. The effect of the original concentration is, therefore, purely mechanical in nature.

5. At low temperatures swollen gels in equilibrium with water tend to approximate to a concentration of 10 %, regardless of the original concentration of the gel.

6. In similar conditions gels which have been dried contain, at equilibrium, less water than those which have not been dried.

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CLXXII. THE EFFECTS OF CYANIDE AND SOME OTHER SALTS ON FERMENTATION BY YEAST PREPARATIONS.

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Particular interest is attached to the effect of HCN and cyanides on alcoholic fermentation because it is possible that one or other of the component reactions bringing about fermentation might be catalysed by metals and hence be specifically inhibited by cyanide.

Complete inhibition of fermentation with yeast juice by 1·2 % HCN (approx. $M/2$) was observed by Buchner, Buchner and Hahn [1903] but the minimum effective concentration had not been determined.

The effect of cyanides on the rate of hexosediphosphate formation by maceration extract and dried yeast at p_H 8·4 was examined by Neuberg and Kobel [1925]. They found that the reaction was delayed but finally went to completion, the p_H changing to about 6·8. Provided the p_H was not allowed to fall during the reaction, however, there was no observed effect with dried yeast. They were unable to give a definite explanation of the phenomenon.

Further, Neuberg and Perlman [1925] showed that 0·01–0·001 M cyanide slowed down the production of alcohol from sugar by maceration extract and dried yeast and in freshly fermenting yeast preparations completely inhibited it.

Warburg [1925] also investigated the effect of cyanide on alcoholic fermentation to decide whether it acted as a narcotic or by virtue of a chemical reaction. He found with maceration extract and living yeast that 0·01 N HCN diminished the rate of fermentation by 90–50 %, whereas with acetonitrile, a typical weak narcotic, a concentration of $2M$ was necessary to bring about a similar effect. He concluded that HCN was not acting as a narcotic.

All these authors found that the concentrations required to bring about these effects were greater than those required to bring about partial or complete inhibition of reactions catalysed by metals, which are of the order of 0·001–0·0001 M.

The yeast preparations, zymin and dried yeast, when mixed with a solution of either glucose or fructose do not begin to ferment immediately. The length of this delay during which there is no production of carbon dioxide varies with the volume of the mixture [Harden and Young, 1911] and is termed

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the induction period. It has now been found that the addition of KCN to the mixture lengthens this period; increasing concentrations of KCN producing a progressively longer induction period until, with sufficiently great concentrations, no fermentation occurs.

Experimental.

Effect of cyanide.

In determining the effect of KCN on the fermentation of glucose by yeast preparations, it was found advisable, because of the gradual removal of HCN gas from the system with the evolution of carbon dioxide, to use the apparatus of Slator [1906], in which fermentation is carried out at almost constant volume and at a reduced pressure.

Estimation of the cyanide content of three similar fermentation mixtures, (*a*) immediately after mixing, (*b*) and (*c*) after fermentation, yielded respectively 96, 89 and 86 % of the KCN added, so that the KCN does not suffer any serious loss.

A uniform method of mixing was adopted. The required volume of water was added to the glucose and yeast preparation and the whole saturated with CO_2. To this KCN was added, and the bottles were connected to the manometer with the taps shut off and then shaken; this ensured saturation with CO_2 without loss of HCN. The bottles were then placed in a thermostat at 30° and allowed to remain for 5 minutes until temperature equilibrium was attained. The pressure was then reduced and after shaking two or three times during a period of 5 minutes the initial reading was taken; the bottles were then shaken and read every 5 minutes. The period elapsing between the time of placing the bottles in the bath and the time when the rate of production of carbon dioxide became equal to the final steady rate was taken as the induction period.

The p_H of the mixture was not seriously affected by the addition of the concentrations of KCN used in these experiments and varied between 5·6 and 6·0, determined by the capillator method.

Fermentation. In fermentation mixtures with zymin, dried yeast and maceration extract, it was found that the presence of cyanide after lengthening the induction period

(*a*) slowed down the rate of attainment of the maximum rate in the presence of phosphates;

(*b*) did not affect the maximum rate attained;

(*c*) lowered the basal rate of fermentation (*e.g.* by 18 % with 0·006 *M* KCN; by 36 % with 0·01 *M* KCN) see Fig. 1.

In fermentations carried out with yeast juice, cyanide produced an induction period which was otherwise absent. This delay was followed by an enhanced rate falling to a basal rate, which was lower than in similar mixtures without cyanide.

These results show that cyanide chiefly affects the mechanism for the liberation of phosphate from hexosephosphate on which the basal rate depends, whilst the processes which occur in presence of free phosphate are unaffected.

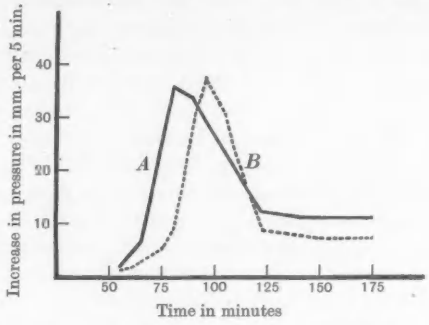

Fig. 1. *A* = Fermentation of control mixture after 65 mins. induction period.
B = Fermentation of mixture containing 0·008 *M* KCN after 225 mins. induction period.

Induction period. Zymin (yeast treated with acetone). Fermentation with 2 g. zymin and 2 g. glucose in 15 cc. volume showed a well-marked induction period. The addition of 0·0007 *M* KCN was ineffective, but from concentration 0·006 *M* KCN upwards the induction period was prolonged to an extent varying with the concentration (Table I, Exp. I, 1, 2, 5; 8, 9). As described by Harden and Macfarlane [1928] the addition of specific concentrations of

Table I.

No. of exp.	Yeast preparation	KCN *M*	K_2HPO_4 *M*	Potassium acetate	Potassium hexose-diphosphate	Induction period, minutes
I 1	Zymin (Z 11)	—	—	—	—	35
2	,,	0·008	—	—	—	55
3	,,	—	0·02	—	—	15
4	,,	0·008	0·02	—	—	35
5	,,	0·012	—	—	—	60
6	,,	—	—	0·2	—	10
7	,,	0·012	—	0·2	—	25
8	(Z 12)	—	—	—	—	25
9	,,	0·033	—	—	—	120
10	,,	—	—	—	0·005	10
11	,,	0·033	—	—	0·005	40

either K_2HPO_4, potassium acetate or potassium hexosediphosphate to a mixture of zymin and glucose solution shortened the induction period. In the presence of KCN and one of these salts the induction period was intermediate between that produced by KCN alone and the shortened one due to the salt action (Table I, Exp. I, 2, 3, 4; 5, 6, 7; 9, 10, 11).

Autofermentation. The autofermentation of zymin was also delayed in the presence of cyanide. 2 g. zymin (Z 14) in 30 cc. water commenced to ferment in 60 minutes but was delayed 25 minutes by 0·01 *M* KCN. With 2 g. zymin

(Z 18) in 20 cc. water the delay was increased from 40 to 55 minutes by the addition of 0·013 *M* KCN.

Dried yeast. This preparation behaved in a similar manner to zymin. 2 g. dried yeast and 2 g. glucose in 15 cc. volume showed an induction period of 45 minutes and this was progressively increased by the addition of KCN, being *e.g.* 75 minutes with 0·017 *M* and 100 minutes with 0·033 *M* KCN. Salts had the same effect on the induction period as with zymin.

Maceration extract (Table II). This shows an induction period [Lebedev, 1912] which to some extent resembles that shown by zymin, in as much as it is abolished by hexosediphosphate [Meyerhof, 1918]. The effect of KCN on this was investigated in the same manner as with zymin. Two methods of preparation of maceration extract were employed and the activities of the two solutions were found to differ. Using a dried yeast prepared from a Dutch baker's yeast Lebedev's [1911] method gave a less active solution than the more recent method of Hägglund and Rosenqvist [1927], according to which maceration was carried out in three parts of 0·15 *M* K_2HPO_4 or Na_2HPO_4 solution for 4 hours. This latter preparation had a shorter induction period than the former. The experimental mixtures consisted of 15 cc. maceration

Table II.

Maceration extract	Hexose-diphos-phate *M*	KCN *M*	K ace-tate *M*	Na_2HAsO_4 *M*	K_2SO_4 *M*	KCl *M*	Induction period minutes
Dy. 7 in water	—	—	—	—	—	—	No fermentation
,,	—	0·005	—	—	—	—	,,
,,	0·001	—	—	—	—	—	20
,,	0·011	0·005	—	—	—	—	145
,,	0·011	0·009	—	—	—	—	260
Dy. 9 in water	—	—	—	—	—	—	70
,,	0·005	—	—	—	—	—	15
,,	—	—	0·1	—	—	—	148
,,	0·005	—	0·1	—	—	—	30
,,	—	—	—	0·0025	—	—	100
,,	0·005	—	—	0·0025	—	—	30
,,	—	—	—	—	0·033	—	110
,,	—	—	—	—	—	0·075	No fermentation
,,	0·005	—	—	—	—	0·075	26
Dy. 11 in phos-phate solution	—	—	—	—	—	—	45
	—	0·015	—	—	—	—	No fermentation
,,	0·0033	—	—	—	—	—	15
,,	0·0033	0·015	—	—	—	—	100

extract and 2 g. glucose. The addition of KCN (0·005–0·015 *M*) to the mixture inhibited fermentation, which was not observed during 20 hours. When, however, hexosediphosphate was added at the same time as the cyanide fermentation took place, the induction period being always greater than in the presence of hexosediphosphate alone. The presence of Na_2HAsO_4 which prolongs the induction period with zymin [Harden and Macfarlane, 1928] also prolongs it with maceration extract and to some extent counteracts the shortening effect of hexosediphosphate. Such salts as KCl, K_2SO_4 and potassium acetate, which accelerate the onset of fermentation with zymin, delay

the onset with maceration extract both alone and in presence of hexosediphosphate.

By substituting sucrose for glucose Meyerhof [1918] found that the induction period of maceration extract was somewhat reduced. With the preparations used in the present experiments this only happened in the more active extracts macerated in phosphate solution, the induction period being shortened by 20 minutes.

It appears that the salts which diminish the induction period of zymin and dried yeast prolong it with maceration extract. Hexosediphosphate is the one exception to this, being the only substance tested which accelerated the onset of fermentation with maceration extract.

Yeast juice (Table III). Yeast juice together with glucose shows no induction period, but the addition of KCN of sufficient concentration produces

Table III.

Yeast juice	KCN *M*	Induction period, minutes
In all cases	—	0
Preparation No. 3	0·075	50
„ „ 11	0·075	20
„ „ 5	0·075	35
„ „ 5	0·10	No fermentation
„ „ 6	0·075	10
„ „ 6	0·10	25
„ „ 14	0·13	55

one. The length of the induction period varies with the concentration of KCN and with each sample of juice.

Changes in the form of combination of phosphorus during the induction period of yeast juice and maceration extract.

Yeast juice. To investigate any change in the form of combination of phosphorus that might be taking place during the induction period produced by the presence of KCN, flasks containing similar mixtures were placed in the thermostat and removed one at a time at intervals during the induction period. The action was stopped immediately by the addition of trichloroacetic acid up to 3 % and the phosphate estimations were carried out in the filtrate by the modified Briggs method (Table IV). The mixture consisted of 15 cc. yeast juice and 2 g. glucose (Exp. IV, 1–6).

During a short induction period produced by the presence of cyanide the acid-soluble organic phosphorus increased in amount at the expense of the inorganic phosphorus as esterification took place.

Exp. IV, 7–13. On the other hand, during the early part of a long induction period produced by KCN, hydrolytic action was observed, the acid-soluble inorganic phosphorus increasing in amount at the expense of the organic phosphorus. Esterification, indicated by increase in acid-soluble organic phosphorus, took place towards the end of the induction period.

During the induction period the total acid-soluble phosphorus remained unaltered.

Exp. IV, 2, 6, 8, 13. After fermentation had proceeded for some time and a steady rate had been established, the acid-soluble inorganic phosphorus was lower in the presence of cyanide than in its absence. At the same time a depressed basal rate was observed.

Maceration extract. In maceration extract the acid-soluble total phosphorus is practically all present as inorganic phosphorus.

Table IV, Exp. IV, 14, 15, 16. A mixture of 10 cc. of maceration extract made from Dy. 11, with 2 g. glucose in 15 cc. volume fermented after 45 minutes. During this time the total acid-soluble phosphorus (60·0 mg.) remained unchanged and the inorganic phosphorus (58·95 mg.) slowly decreased.

Table IV.

No. of exp.	Yeast preparation	Time after mixing (mins.)	KCN *M*	Hexose-diphos-phate *M*	Acid-soluble phosphorus: Total mg.	Acid-soluble phosphorus: Inor-ganic mg.	Acid-soluble phosphorus: Organic mg.	Fermentation commenced
IV 1	Yeast juice 13	0	—	—	76·91	23·51	53·40	Immediately
2	,,	70	—	—	,,	1·20	75·71	—
3	,,	0	0·05	—	,,	23·51	53·40	—
4	,,	10	,,	—	,,	19·52	57·39	—
5	,,	15	,,	—	,,	17·92	58·99	After 15 mins.
6	,,	85	,,	—	,,	1·11	75·80	—
7	Yeast juice 14	0	—	—	75·63	9·21	66·42	Immediately
8	,,	60	—	—	,,	1·92	73·71	—
9	,,	0	0·13	—	,,	9·21	66·42	—
10	,,	25	,,	—	,,	13·22	52·41	—
11	,,	35	,,	—	,,	15·43	60·20	—
12	,,	50	,,	—	,,	9·46	66·17	After 50 mins.
13	,,	115	,,	—	,,	1·52	74·11	—
14	Maceration extract	0	—	—	60·00	58·95	1·05	—
15	(Dy. 11)	20	—	—	,,	56·40	3·60	—
16	,,	40	—	—	,,	53·10	6·90	After 45 mins.
17	,,	0	0·015	—	58·80	58·80	—	—
18	,,	20	,,	—	,,	57·75	1·05	—
19	,,	40	,,	—	,,	57·30	1·50	—
20	,,	70	,,	—	,,	57·15	1·65	—
21	,,	130	,,	—	,,	55·95	2·85	No fermentation
22	,,	0	—	0·0033	64·65	59·10	5·55	—
23	,,	7	—	,,	,,	59·55	5·10	—
24	,,	12	—	,,	,,	55·95	8·70	After 15 mins.
25	,,	0	0·015	0·0033	63·75	58·80	4·95	—
26	,,	20	,,	,,	,,	59·10	4·65	—
27	,,	40	,,	,,	,,	58·80	4·95	—
28	,,	70	,,	,,	,,	55·95	7·80	—
29	,,	90	,,	,,	,,	55·65	8·10	—
30	,,	100	,,	,,	,,	55·50	8·25	After 100 mins.

Exp. IV, 17–30. Fermentation was completely inhibited in such a mixture by 0·015 *M* KCN. In 130 minutes the acid-soluble inorganic phosphorus decreased by 2·85 mg. The total phosphorus remained unaltered. The addition of 0·0033 *M* potassium hexosediphosphate shortened the induction period considerably from 45 to 15 minutes in the control without cyanide, during which time there was a small amount of esterification shown by a decrease of 3·15 mg. in the acid-soluble inorganic phosphorus. A similar decrease

(3·3 mg.) took place during the 100 minutes' induction period of a mixture containing 0·015 *M* KCN as well as 0·0033 *M* potassium hexosediphosphate. In all these cases the total acid-soluble phosphorus remained constant and no change occurred except a slight esterification towards the end of the induction period.

The induction period of zymin.

In an attempt to arrive at an explanation of the cause of the induction period before fermentation with zymin, the required amount of zymin was ground up in a 12-hour old glucose solution and the desired quantities of the mixture were pipetted into test-tubes which were placed in a thermostat. Determinations of the optical rotation, reducing power and phosphates were made in the trichloroacetic acid filtrates at zero time and subsequently at intervals during the induction period. The reducing power as glucose of the mixture was determined by the Hagedorn-Jensen method (Table V). During the early part of the induction period of 100 minutes of a mixture of 3 g. zymin (Z 18) + 48 cc. glucose solution in a total volume of 60 cc. there were only

Table V.

Mixture	Time after mixing	Optical rotation α_{5461}	Reducing power as glucose mg./1 cc.	Fermentation commenced
3 g. Z 18 + 48 cc. glucose solution in total volume 60 cc.	0	+2·90	44·4	—
	70	+2·81	45·0	—
	80	+2·81	44·6	—
	90	+2·75	42·6	After
	100	+2·68	42·4	100 mins.

slight changes observed in either optical rotation or reducing power. As fermentation commenced these values slowly decreased, as might be expected. In mixtures with 0·13 *M* and 0·2 *M* potassium acetate, which shorten the induction period, and with 0·015 *M* KCN or 0·0067 *M* Na_2HAsO_4 which lengthen the induction period, and with fructose substituted for glucose the same order of changes occurred.

Phosphorus estimations. As before, these estimations were carried out according to the modified Briggs method. On investigating the changes occurring during the induction period when no carbon dioxide was given off, it became evident that the form of combination of the phosphorus altered in an arresting manner [see also Macfarlane, 1931].

Table VI, Exp. VI, 1–7. In the control mixture of 3 g. zymin (Z 18) + 48 cc. glucose solution in a total volume of 60 cc. there was an increase in the total acid-soluble phosphorus from 33·00 mg. to 53·66 mg. during the 100 minutes' induction period. During the early part of the induction period there was an almost corresponding increase in the acid-soluble inorganic phosphorus, followed by a decrease towards the time of commencement of fermentation. The acid-soluble organic phosphorus remained almost constant

Table VI.

No. of exp.	Mixture	Time after mixing minutes	Acid-soluble phosphorus			Fermentation commenced
			Total P mg.	Inorganic P mg.	Organic P mg.	
VI 1	3 g. Z 18 + 48 cc. glucose	0	33·00	10·63	22·37	
2	solution in total volume	20	39·60	18·74	20·86	
3	60 cc.	40	44·63	23·70	20·98	
4		70	47·85	19·47	28·38	
5		80	50·95	13·73	37·22	
6		90	52·80	5·87	46·93	After
7		100	53·66	4·69	48·97	100 mins.
8	3 g. Z 18 + 48 cc. glucose	0	38·35	10·97	27·38	
9	solution + 0·13 *M* potas-	25	46·79	16·90	29·89	
10	sium acetate in total	35	49·24	5·21	44·03	After
11	volume 60 cc.	40	49·96	5·08	44·88	45 mins.
12	3 g. Z 18 + 48 cc. glucose	0	46·79	11·15	35·64	
13	solution + 0·2 *M* potas-	7	49·43	13·00	36·43	
14	sium acetate in total	14	50·36	6·14	44·22	
15	volume 60 cc.	21	50·56	3·76	46·80	After
16		28	50·82	3·78	47·04	25 mins.
17	3 g. Z 18 + 48 cc. glucose	0	34·91	10·63	24·28	
18	solution + 0·01 *M* KCN	20	38·74	14·32	24·42	
19	in total volume 60 cc.	40	41·38	21·12	20·26	
20		70	46·46	26·93	19·53	
21		100	50·86	32·57	18·29	
22		130	52·07	38·48	13·59	
23		150	52·07	38·15	13·82	
24		170	52·20	35·64	16·56	
25		190	52·58	28·18	24·40	
26		210	52·67	26·80	25·87	After
27		230	52·80	13·46	39·34	230 mins.
28	3 g. Z 18 + 24 cc. glucose	0	40·25	11·02	29·23	
29	solution in total volume	25	45·46	11·88	33·58	
30	30 cc.	35	49·39	7·70	41·69	After
31		40	50·29	7·09	43·20	40 mins.
32	3 g. Z 18 + 24 cc. glucose	0	44·03	11·48	32·55	
33	solution + 0·015 *M* KCN	25	44·28	21·17	23·11	
34	in total volume 30 cc.	35	46·55	23·87	22·68	
35		55	48·78	28·55	20·23	
36		70	52·20	28·12	24·08	
37		80	52·20	15·52	36·68	After
38		100	52·34	7·20	45·14	100 mins.

Z 18 = Zymin No. 18

for the first part of the induction period and then rapidly increased as esterification took place.

Exp. VI, 8–11. In the 45 minutes' induction period in the presence of 0·13 *M* potassium acetate in a similar mixture the total acid-soluble phosphorus increased to almost the same value as in 100 minutes in the control. The absolute increase was, however, less because the total acid-soluble phosphorus at mixing was higher in the presence of acetate than in its absence. As in the control, the acid-soluble inorganic phosphorus at first increased and then later decreased. The organic phosphorus remained almost constant for the first 25 minutes and then increased.

Exp. VI, 12–16. In the presence of 0·2 *M* potassium acetate the induction period of this mixture was further shortened to 25 minutes and when fermentation began the total acid-soluble phosphorus was again approximately the

same; the absolute increase being again less owing to the higher original total acid-soluble phosphorus. Both the inorganic and organic phosphorus behaved as in the former case with acetate present.

If the changes observed in the concentration and rate of extraction of the total acid-soluble phosphorus in zymin bear any relation to the length of the induction period it might be expected that the increase in the rate of extraction of phosphorus coincident with a shorter induction period, which is shown to take place in presence of acetate, would be reversed with cyanide, and this was found to be the case.

Exp. VI, 17–27. The presence of 0·01 *M* KCN in a mixture of 3 g. zymin (Z 18) + 48 cc. glucose solution in 60 cc. volume prolonged the induction period by 130 minutes. The total acid-soluble phosphorus values at mixing and at the onset of fermentation were almost the same as in the control. The absolute increase was therefore identical, but the period required for its production was almost $2\frac{1}{2}$ times as long (see Fig. 2).

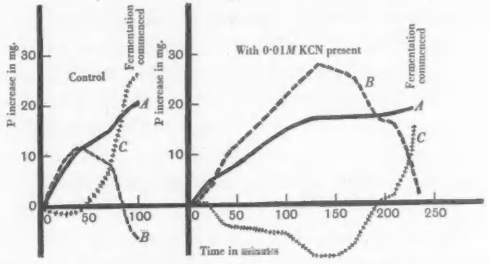

Fig. 2. *A* = acid-soluble total phosphorus. *B* = acid-soluble inorganic phosphorus. *C* = acid-soluble organic phosphorus.

Exp. VI, 28–38. This relation was again shown in two mixtures of half the total volume. 3 g. zymin (Z 18) + 24 cc. glucose solution in 30 cc. volume, one with 0·015 *M* KCN and one without. The absolute increase of total acid-soluble phosphorus in the two mixtures was about the same, but its production required 60 minutes longer in the mixture containing cyanide.

As shown in the experiments with and without potassium acetate present, the amount of increase of acid-soluble inorganic phosphorus during the first part of the induction period was approximately the same as that of the total phosphorus. The extra phosphorus derived from the zymin, which was estimated as acid-soluble inorganic phosphorus may have been present in the zymin in that form or as an ester which was hydrolysed; there was, however, no direct evidence of this hydrolysis. On the other hand, in the presence of cyanide the increase in inorganic phosphate was greater than the increase in total phosphorus, which indicates that hydrolysis at the expense of the organic phosphorus, which gradually decreased, was occurring. Esterification occurred towards the end of the induction period as in other cases (see Fig. 2).

In this present set of experiments the only evidence of hydrolysis of organic phosphorus throughout the early part of the induction period of zymin fermentation was obtained in the experiments with cyanide.

This observation that fermentation takes place when an approximately constant concentration of total acid-soluble phosphorus derived from the zymin is reached, throws some light on the nature of the effect of salts. In all the experiments with 3 g. zymin (Z 18) in 60 cc. volume both in the presence and absence of added salts, fermentation began when the concentration of total acid-soluble phosphorus was between 49·0 mg. and 54·0 mg. At mixing the acid-soluble total phosphate was 33·00 mg. in the control, 38·35 mg. in the presence of 0·13 *M* potassium acetate and 46·79 mg. with 0·2 *M* potassium acetate. It was thus seen that of the mixtures in which the same concentration of total phosphorus was reached before fermentation commenced, those in which the total phosphorus at mixing was higher had the shorter induction periods.

In another mixture of 3 g. zymin (Z 18) in 30 cc. volume, *i.e.* half the volume in the former experiments, the total at mixing was 40·25 mg. increasing to 50·29 mg. in 40 minutes when fermentation began. The length of this induction period and the total acid-soluble phosphorus both at mixing and when fermentation began are comparable with those of the mixture in double the volume containing 0·13 *M* potassium acetate. The presence of this salt to some extent counteracts the effect of dilution.

A comparison of the two control mixtures, one in 60 cc. volume and the other in 30 cc., further illustrates this point. At mixing, the total acid-soluble phosphorus of the more dilute mixture was 33·00 mg., this was lower than that of the mixture in 30 cc., which was 40·25 mg. They both began to ferment when the total phosphorus was approximately the same (50·29 mg. in 30 cc. and 53·66 mg. in 60 cc.), but the time taken to arrive at this concentration was 60 minutes longer in the more dilute mixture. Hence dilution retarded the accumulation of phosphate from zymin and increased the induction period.

There still remained a certain amount of phosphorus in the zymin, even after 3 hours' incubation, for the highest value of total acid-soluble phosphorus obtained was never as high as the absolute total phosphorus found. Thus the total phosphorus of 1 g. zymin (Z 18) was 21·0 mg. and the highest total acid-soluble found was 17·5 mg.

Inorganic phosphorus during the basal rate.

It has already been shown that the basal rate of fermentation of sugar solution with zymin was reduced in the presence of cyanide. The acid-soluble inorganic phosphorus of a mixture of 2 g. zymin (Z 14) + 2 g. glucose in 30 cc. volume fermenting at a steady rate was 1·70 mg. By the presence of 0·007 *M* KCN this was reduced to 1·40 mg. and to 1·26 mg. by the presence of 0·01 *M* KCN. These results again show that the presence of cyanide has

interfered with the mechanism by which phosphate is obtained from hexose-diphosphate.

Discussion.

It is inferred, partly from the negative results obtained with regard to the optical rotation and reducing powers of the mixtures and partly from the positive evidence of changes in the form of combination of the phosphorus, that the duration of the induction period of fermentation mixtures with zymin is dependent on changes taking place in the zymin. It is uncertain whether the actual release of phosphate or whether its condition of availability is the necessary factor to bring about the commencement of fermentation. It is also conceivable that the liberation of phosphate may be accompanied by other secondary changes in the zymin by which another substance or group of substances directly influential in bringing about fermentation is made available. At present it is obscure how the liberation of phosphate is hastened or retarded, but the question of an alteration of the permeability of the cell wall of the acetone yeast preparation arises. Potassium acetate which shortens the induction period increases the rate at which the available phosphate becomes acid-soluble. In the presence of cyanide, however, which increases the induction period, and decreases the rate of increase of acid-soluble phosphorus, fermentation does not begin when the concentration of total phosphorus is reached which is effective in the control or in the presence of potassium acetate. The cyanide evidently also exerts some other effect. The essential point does not seem to be the accumulation of organic phosphorus, as this is decreasing throughout nearly the whole of the induction period in the presence of KCN and its increase towards the end is probably the result of the fermentation and not the cause of it, there being always a lag between esterification and evolution of CO_2 [Boyland, 1930].

Cyanide and arsenate.

It having being definitely established that both cyanide and arsenate prolong the induction period of zymin and glucose mixtures, it seemed probable that by introducing these salts independently and together into a mixture some light might be thrown on the nature of their separate actions (see Fig. 3). A mixture of 2 g. zymin (Z 18) with 2 g. glucose in 15 cc. had an induction period of 30 minutes. This was prolonged to about 70 minutes in presence of either 0·013 *M* KCN or of 0·0067 *M* Na_2HAsO_4 (called concentration 1). On doubling and trebling these concentrations (concentrations 2 and 3) almost proportional increases in the induction period were observed with KCN, whereas with arsenate the increases were considerably less (Fig. 3, Curves A and B). It seems, therefore, that the mode of action of the two substances is quite different.

In attempting to investigate this point further the two salts were added together to the mixture and the time effects observed. By adding at the same time the original concentrations (concentration 1) of each salt, the

increase in the induction period (curve *C*) was intermediate between those obtained with concentration 2 of the salts separately, and nearer to the time

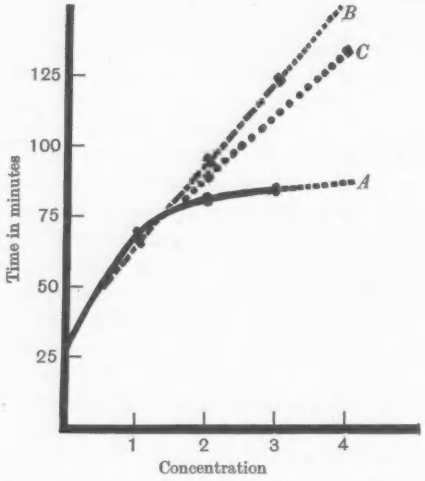

Fig. 3. *A* = Induction period due to Na_2HAsO_4. *B* = Induction period due to KCN. *C* = Induction period due to Na_2HAsO_4 and KCN.

due to the cyanide effect. The addition of 0·026 *M* KCN and 0·0134 *M* Na_2HAsO_4 (concentration 2) together to a mixture produced an induction period, which lies between those which would be expected from the curves of concentration 4 of KCN and Na_2HAsO_4 added separately. The effect was thus found to be more than that of either salt alone but less than the sum of those produced by the two salts alone. At the same time it was more than that produced by the double concentration of arsenate, but less than that due to the double concentration of cyanide. This suggests that interference takes place between the actions of the two substances possibly because they may affect reactions dependent to a certain extent on one another.

Methylglyoxal.

It is supposed that methylglyoxal occurs as an intermediate product of alcoholic fermentation. If this substance is formed towards the end of the induction period as suggested by Boyland [1930], by including it in the original fermentation mixture a shortening of the induction period might be brought about. This has been found to occur in the present experiments with zymin. As the sample of methylglyoxal hydrate[1] used was acid in reaction (p_H 1·6) before each experiment the p_H was adjusted to 6·0–6·2 with Na_2CO_3.

[1] I am indebted to Prof. R. Robinson for this specimen of methylglyoxal.

Table VII.

No. of exp.	Mixture	Methyl-glyoxal *M*	Induction period, minutes	Reduction of Phosphate rate %	Reduction of Basal rate %
VII 1	2 g. Z 18 + 2 g. glucose in	—	60	—	—
2	20 cc. volume	0·01	60	—	—
3		0·01*	—	—	—
4		0·02	10	45	40
5		0·02*	—	25	25
6		0·03	15	60	60
7		0·03*	—	35	38
8		0·05	20	Complete	52
9		0·05*	—	52	52
10		0·1	30	Complete	60
11		0·1*	—	60	60
12	2 g. Z 18 + 16 cc. water	—	25	—	—
13		0·05	15	55	60
14	2 g. Z 18 + 26 cc. water	—	70	—	—
15		0·02*	—	30	33
16		0·03*	—	54	55

* Denotes addition at the end of the normal induction period.

Table VII, Exp. VII, 1, 2, 3, 4. The mixture used was 2 g. zymin (Z 18) and 2 g. glucose in 20 cc., which had an induction period of 60 minutes. The concentration of methylglyoxal which had the maximum time effect and shortened the induction period to 10 minutes was 0·02 *M*. After 65 minutes of fermentation at a reduced rate a maximum rate was reached, which was less than in the control without methylglyoxal. The basal rate was reduced by 40 % in the presence of methylglyoxal.

Exp. VII, 6, 8, 10. Concentrations of 0·03 *M*, 0·05 *M* and 0·1 *M* shortened the induction period and increasingly reduced the basal rate up to 60 % in the case of 0·1 *M*. No rise to a maximum rate occurred in the presence of either 0·05 *M* or 0·1 *M* methylglyoxal.

Exp. VII, 5, 7, 9, 11. To determine the effect on fermentation, similar concentrations to the above were added at the onset of fermentation (*i.e.* after the normal induction period of 60 minutes of the mixture). The evolution of CO_2 decreased with increasing concentration of methylglyoxal, the basal rate being reduced 40 % with 0·02 *M* and 60 % with 0·1 *M* methylglyoxal.

Table VIII. On addition of K_2HPO_4, in optimum concentration (0·09 *M*),

Table VIII.

Mixture	K_2HPO_4 *M*	Methyl-glyoxal *M*	Rate of CO_2 production: Highest	Basal	Phosphate rate (cc. per 5 minutes)	Percentage reduction: Phosphate rate	Basal rate
2 g. Z 18 + 2 g.	0·06	—	12·8	3·2	9·6	—	—
glucose in 15 cc.	0·06	0·035	10·9	2·3	8·6	10·4	28·1
volume	0·09	—	13·6	3·1	10·5	—	—
	0·09	0·02	12·5	2·9	9·6	8·5	6·4
	0·09	0·035	11·5	2·7	8·8	16·1	12·6
	0·09	0·05	9·4	2·1	7·3	30·4	32·2
	0·15	—	13·1	3·4	9·7	—	—
	0·15	0·05	9·0	2·1	7·9	18·5	38·2

together with methylglyoxal in various concentrations at the onset of fermentation, a lower maximum phosphate rate and basal rate were observed than in the absence of methylglyoxal. With 0·02 *M* methylglyoxal the maximum rate was 8·5 % and with 0·05 *M* 43·2 % less than the control, the basal rates of the same mixtures being 6·1 % and 32·2 % lower. In the presence of methylglyoxal the maximum rate was attained about 10 minutes sooner than in its absence.

Exp. VII, 12–16. The autofermentation of zymin (Z 18) in the presence of methylglyoxal showed the same inhibition. With 0·05 *M* methylglyoxal the induction period was shortened from 25 minutes to 15 minutes and the basal rate reduced by 60 %. At the onset of autofermentation the addition of 0·02 *M* and 0·03 *M* methylglyoxal reduced the maximum rates 30 % and 54 % respectively and the basal 33 % and 55 %. There was no indication of fermentation of methylglyoxal as there was a smaller evolution of carbon dioxide in its presence than in its absence.

It appears from these experiments that methylglyoxal exerts an inhibiting influence, either directly or indirectly, both on the process of esterification and on the mechanism which controls the basal rate. At the same time it shortens the induction period.

Summary.

1. In alcoholic fermentation by yeast preparations KCN (minimum concentration 0·006 *M*) slows down the attainment of a maximum rate in the presence of phosphate, without affecting the maximum rate attained. The basal rate of fermentation is lowered.

2. The induction period before the onset of fermentation by yeast preparations (zymin, dried yeast and maceration extract) is increased by cyanide (minimum concentration 0·005 *M*); with yeast juice, cyanide (minimum concentration 0·075 *M*) produces an induction period which is otherwise absent.

3. In the presence of substances such as potassium acetate, potassium hexosediphosphate and dipotassium hydrogen phosphate, which shorten the induction period of zymin and dried yeast, cyanide causes a prolongation of it to a smaller degree than when acting alone.

4. With yeast juice in the presence of cyanide, the total acid-soluble phosphorus remained constant, the organic phosphorus diminished and the inorganic phosphorus increased till near the end of the induction period.

5. With maceration extract the total phosphorus remained constant and there was no change except slight esterification towards the end of the induction period.

6. During the induction period of zymin under various conditions the total and inorganic acid-soluble phosphorus increased by the same amount while the organic phosphorus remained approximately constant till near the end of that period, when it increased as the inorganic phosphorus became esterified.

7. In the presence of cyanide during the first part of the induction period there was a greater increase of acid-soluble inorganic phosphorus than of total phosphorus. This was due to hydrolysis of the organic phosphorus.

8. The optical rotation and reducing power of the trichloroacetic acid filtrates from the fermentation mixtures only showed slight changes until the end of the induction period was approached.

9. In the presence of arsenate, which prolongs the induction period of zymin, cyanide further prolongs it, but the joint effect is less than the sum of the separate effects. The curve connecting increase of induction period with increase of concentration is different for each salt, indicating that their actions are of a different kind.

10. Methylglyoxal shortened the induction period of fermentation with zymin and reduced both the basal rate of fermentation and the rate of esterification.

I desire in conclusion to express my thanks to Prof. A. Harden for his constant advice and help in this work.

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CLXXIII. A SPECTROGRAPHIC ANALYSIS OF HUMAN TISSUES.

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This paper describes the results of a spectroscopic examination of 195 specimens of different human tissues, both healthy and diseased. The object of the investigation was to determine whether any chemical elements were constantly present in the tissues in addition to those already recognised as being universal constituents of protoplasm (C, H, O, N, P, S, Na, K, Mg, Ca, Cl, Fe, Cu) and to discover what variations might occur both between different organs and between healthy and diseased tissues.

Preparation of the material.

A general description of the method has been published by one of us [Ramage, 1929]. Small pieces of the organ were removed and washed in distilled water to free them from blood as far as possible. They were then placed in a steam-oven and dried until of constant weight. In cases where the presence of fat made this difficult, the melted fat was removed by drying them on the ashless filter-paper used for dealing with fluid specimens. The resulting material was then ground to powder in an agate mortar, and a small amount of this powder was used for obtaining the spectrum. Except where it is otherwise stated, the amount of each specimen that was used was 0·05 g. This was rolled in a small piece of ashless filter-paper—weighing about 0·12 g.—cut from Munktell's Swedish paper No. 00 diameter 12·5 cm., and the whole was then burnt in an oxy-coal-gas flame before the slit of a quartz spectrograph, a quartz lens being used to focus the image of the flame on the slit. The spectrum of the flame itself has no metallic lines, but shows a number of lines in the ultra-violet due to water-vapour. The spectrum of the filter-paper shows very weak lines due to sodium, potassium, and calcium, but apart from these is devoid of metallic lines. The spectrograph employed was a Size C Hilger quartz spectrograph, and the photographic plates have been Ilford panchromatic coated on thin glass. Plates 5″ by 4″ suitably placed in the holder cover the region from the red to beyond λ2800, which is that required when no arc is used. In the case of fluids such as blood, 0·1 cc. was transferred to a piece of the filter-paper which had previously been rolled

from the curved edge, dried in the steam-oven, and burnt in front of the spectrograph in the same way as the solid specimens.

Standard solutions were prepared having the following percentage composition: (1) Na, 0·15; K, 0·20; Ca, 0·02; Li, 0·0004; Rb, 0·004; Mg and Fe, 0·05; Cu and Co, 0·005; Mn and Ni, 0·002; Cd, 0·10; P, 0·50; and (2) Sr, 0·002; Ag, 0·0025; and Pb, 0·05. The brilliance of the lines obtained on burning papers charged with the standard solutions naturally varies with the amount of solution used, and since it is possible to obtain the photographs of 16 spectra on the one photographic plate, four of these were devoted to the standard solutions, each in amounts of 0·02, 0·05, 0·1 and 0·2 cc. In this way, by matching the intensity of the lines of an element in a specimen with that in the different strengths of standard solution, the method can be put to a quantitative as well as a qualitative use.

Though figures are given throughout this paper, it is not desired to lay stress at present on the quantitative nature of the investigation, the original purpose of which was a qualitative survey of the metallic constituents of the various tissues. Only the four strengths of standard solution already mentioned were employed, and in consequence, where the brightness of the lines of a particular element is less than that shown by the element in the weakest strength of the standard solution, a "trace" of the element in question has usually been said to be present. In using the method further for quantitative purposes, either a more extensive range of variation of the strengths of standard solution could be employed, or the standard weight of specimen burnt (0·05 g. here) could be altered, so that the lines could be accurately matched.

Except in the case of blood, all the percentages given are in terms of the dry weight of the tissue. Where the term "trace" is used, the element in question is present in a percentage of less than the following, which are the percentages represented (when 0·05 g. of specimen is used) by lines matching those formed by the burning of 0·02 cc. of standard solution.

	%		%
Sodium	0·06	Manganese ...	0·0008
Potassium ...	0·08	Iron	0·02
Lithium	0·00016	Silver	0·001
Calcium	0·008	Strontium ...	0·0008
Rubidium ...	0·0016	Lead	0·02
Magnesium ...	0·02	Phosphorus ...	0·20
Copper	0·002		

By this method the following elements in addition to those mentioned above may be readily detected when present in 0·05 g. of a specimen: caesium, barium, cadmium, gallium, indium, thallium, bismuth, chromium, cobalt, nickel, ruthenium, rhodium, palladium and iridium. Other elements have been described as occurring in plant or animal tissues which are not included in this list, of which the more important are zinc, aluminium, tin and

titanium. These elements can be detected when an arc spectrum is employed, and in a recent paper by Zbinden [1930] were all described as occurring in human tissues. Zinc has been described by Bodansky [1921] as a normal constituent of the brain. The reason why these elements cannot be detected directly by the method employed here has already been given by Fox and Ramage [1931], *viz.* "that the thermal or electrical conditions of the flame are not sufficiently intense either (*a*) to reduce the oxides in adequate quantities or (*b*) when small quantities only are present, to excite the vibrations producing the lines with enough energy to photograph them, placed, as most of them are, in the further portion of the ultra-violet region of the spectrum, *i.e.* beyond wave-length 3100." The arc spectrum employed by Zbinden, making use of carbon electrodes, while more sensitive, introduces a risk of error due to impurities in the carbons *etc.*, which is not present in our method.

In view of the fact that the presence and distribution of sodium, calcium, potassium and magnesium through the body is already well known, these metals are not referred to except where they show some striking anomaly.

The cases from which tissues were taken for examination may be summarised as follows:

S. Male, age 77. Death from cerebral haemorrhage, due to arteriosclerosis. The heart was enlarged and adherent to the pericardium. The pericardial adhesions were calcified in places, and the mitral valve also showed calcified nodules.

F.M. Female, age 69. Death from cholangitis, following chronic inflammation of the gall-bladder.

K.T. Female, age 2. Death from acute broncho-pneumonia.

A.B. Male, age 35. Death from pneumonia following operation for a perforated duodenal ulcer.

G.E. Male, age 53. Squamous-celled carcinoma of the oesophagus.

N.C. Female, age 27. Death from acute tubercular broncho-pneumonia. Both lungs showed widespread tubercular deposits, without obvious caseation. The thyroid gland contained a small adenoma.

M.R. Female, age 7. Acute broncho-pneumonia.

M.L. Male, age unknown. Advanced chronic interstitial nephritis.

E.C. Female, age 5. Death from diphtheritic toxaemia.

G.C. Male, age 9 months. Acute broncho-pneumonia.

R.M. Male, age 61. Death from uraemia.

H. Male, age 25. Miliary tuberculosis.

Foetus D, 24 weeks development. This was a normal foetus, which was removed at operation by Mr Maslen Jones, owing to heart disease of the mother.

Foetus D (*a*), 24 weeks development. Abortion of unknown origin.

Foetus F, female, full-term development. Stillborn.

Foetus (10), foetus of 10 weeks' development. Abortion.

L. Female, age 20. Chronic lymphatic leukaemia.

J. Male, age 72. Advanced atrophic cirrhosis of the liver, which was probably alcoholic in origin.

E.B. Male, age 49. Death from tuberculosis with advanced silicosis of the lung.

P. Male, age unknown. Advanced silicosis of the lung.

L (2). Female, age unknown. Myeloid leukaemia, death occurring while 26 weeks pregnant.

Foetus L (2). The foetus of the above case.

B.P. Age 9 months. Death from miliary tuberculosis.

G.P. Owing to the difficulty of freeing the human tissues removed at *post-mortem* from all their contained blood, this was done in the case of a guinea-pig. The animal was killed by bleeding,

and immediately after death a cannula was inserted into the aorta and the animal transfused with distilled water. The organs, after being dissected out, were minced and again washed in glass-distilled water before being placed in the drying oven.

Description of the various tissues.

Table I. *Liver.*

Patient	Copper	Manganese	Rubidium	Silver
S.	Trace	0·0004	0·002	Trace
F.M.	0·0015	0·0004	0·002	Trace
A.B.	0·002	0·0005	0·003	Trace
G.E.	0·003	0·0008	0·004	Trace
N.C.	0·0015	Trace	0·0016	—
Foetus D	0·035	0·001	0·004	Merest trace
Foetus (10)	0·015	Trace	None	Merest trace
J.	0·02	0·0008	0·0016	—
E.C.	0·002	0·0008	0·0024	Trace
Foetus F	0·025	0·001	0·0016	Trace
Foetus D (*a*)	0·03	0·0004	0·002	—
G.C.	0·002	Trace	0·002	—
R.M.	0·02 +	0·0008	0·004	—
Foetus L (2)	0·02 +	Trace	0·0016	—
L (2)	0·002	0·0008	0·0016	Trace
B.P.	0·02	Trace	0·0024	—
G.P.	0·0075	0·002	0·004	—

Liver. The iron spectrum was rich in all the specimens examined, including that of the transfused guinea-pig. The lines of Na, K, Ca and Mg reveal no unusual features, except that the liver of the 10-weeks foetus was very high in Ca and very low in Mg and K. Copper was present throughout, and, in general, the lines indicate a higher concentration in this organ than in any other region of the body; *e.g.* in the transfused guinea-pig, 0·0075 % in the liver, as against slightly less than 0·002 % for the other organs of this animal that were examined. The most prominent feature of the distribution of copper in the liver as shown by Table I is the high Cu content of the foetal liver, the lowest amount, found in the 10-weeks specimen (0·015 %), being some three times as much as that of the average figure (0·0045 %) for the adult livers. This particular specimen was in an advanced state of autolysis when dissected out, and it was only possible to obtain 34 mg. of dry tissue for burning. The other four foetal specimens have an average of 0·0275 %. The difference is strikingly shown by the case of L (2) where the maternal liver had 0·002 % Cu and the foetal liver more than 0·02 % Cu. That there is considerable variability in the rate of dispersion of copper from the liver after birth is shown by the difference between the Cu contents of the livers of two 9 months old babies (G.C. 0·002 % and B.P. 0·02 %). The high Cu content of the foetal liver is probably connected with its blood-forming functions in this stage of life, since it has been shown by Waddell *et al.* [1928, 1929] that the presence of this element is necessary for the formation of haemoglobin.

Specimens J. and R.M. are of interest, since these were the only adult livers to give copper lines of an intensity comparable with those found in the

foetus. In the case of J. the liver was examined in the first place with the idea that it might contain an abnormal amount of manganese, in view of the facility with which cirrhosis of the liver can be produced in experimental animals by this metal. The manganese line was, however, weak, but the copper lines were very strong (0·02 %). Whether the presence of this large amount of copper bears any relation to the development of the disease is uncertain. Schonheimer and Oshima [1929] state that cirrhosis of the liver may occur with a normal amount of copper. The case of R.M. (uraemia) is of importance in the reverse connection, since the liver contained slightly more than 0·02 % of copper, while the organ itself, though large, was normal in appearance except for some fatty degeneration, and showed no sign of cirrhosis. Manganese was found in every liver, and in five instances the amount present was less than 0·0008 %. The organs of the baby G.C. who died at the age of 9 months from broncho-pneumonia, had, in general, a low content of copper and manganese, which raises the interesting speculation as to whether a deficiency of these metals plays any part in lowering resistance towards infection. The transfused guinea-pig gave the strongest spectrum (0·002 %) while among the human specimens foetus D and foetus F had the greatest amount (0·001 %). As with copper, the liver apparently has a greater need for manganese during foetal life than after birth. The amount of manganese was low in both foetus (10) and foetus D (*a*), but both of these died from unknown causes, while foetuses F and D were healthy.

Rubidium was present in every liver except that of the 10-weeks foetus. The lines were generally strong, though there were considerable individual variations, the greatest amount in the adults (0·004 %) being found in G.E. (carcinoma of the oesophagus), R.M. (uraemia) and in the liver of foetus D.

Traces of silver occurred in 9 of the 17 specimens of liver. A trace of lead was found in the case of G.E. and the liver of the 10-weeks foetus was unique in containing a trace of strontium. It is worthy of note that 3 of the 5 foetal livers contained traces of silver.

Gall-bladder and bile. Four specimens were examined. (Gall-bladders G.E. and foetus F, bile from foetus F, and both gall-bladder and bile were examined together from foetus D. The bile was dried before examination.)

The gall-bladder of G.E. had strong Ca lines, presumably the result of age. The Cu lines were strong in both gall-bladder and bile of foetus F, the gall-bladder containing 0·0075 % and the bile 0·02 %.

The merest trace of manganese was found in the gall-bladder of G.E., while in foetus F the bladder gave a strong line (0·0015 %) and in the dried bile the metal was present in even larger amount (0·008 %). Faint lines of rubidium were found in all the specimens. The bile of foetus F showed traces of silver, this metal being also found in the liver.

Pancreas. From Table II it will be seen that copper was present in every specimen examined, but that in only one case, that of the full-term foetus, did it rise to 0·002 % of the dry weight. Manganese was found in all cases,

and also rubidium, the lines of which are visible in every spectrum of the pancreas, but nowhere represent a value of more than 0·0024 %. The rubidium lines are weakest in the foetal specimens. Traces of silver were found in three cases. The strength of the manganese lines is the most striking feature of the examination of this organ.

Table II. *Pancreas.*

Patient	Copper	Manganese	Rubidium	Silver
S.	Trace	0·0008	0·0016	Trace
F.M.	Trace	Trace	Trace	Trace
A.B.	Trace	0·001	0·0016	Trace
G.E.	0·002 (almost)	0·0008	0·0024	—
N.C.	Trace	0·0008 (almost)	0·0016	—
Foetus D	0·002	0·0008 (almost)	0·0016	—
Foetus F	0·002	0·001	0·002	—
Foetus D (*a*)	Trace	Trace	Trace	—
G.C.	Trace	0·0008	0·0016	—
R.M.	Trace	0·0008	0·0024	—

Table III. *Spleen.*

Patient	Copper	Manganese	Rubidium	Silver	Other elements
S.	Trace	—	0·0016	—	—
L.	Trace	—	0·0016	—	—
F.M.	Trace	—	0·0016	—	—
K.T.	0·002	0·0008	0·001	—	Trace of lithium
A.B.	Trace	—	0·0024	—	—
G.E.	Trace	—	0·004	Trace	—
N.C.	Trace	Trace	0·0024	—	—
E.C.	Trace	—	0·004	—	—
Foetus D	Trace	Trace	0·005	—	—
Foetus F	0·002	—	0·004	—	—
Foetus D (*a*)	Trace	—	0·004	—	—
G.P.	Trace	Trace	0·006	—	—

Spleen. As was expected, the iron lines were very strong in the spectra of this organ. The magnesium lines showed nothing remarkable. Copper was present in every specimen, reaching a concentration of 0·002 % in K.T. (a girl age 2) and in the full-term foetus. Manganese was only found in 5 out of the 12 spleens examined. In the highest (K.T.) it only reached 0·0008 %, while in all the others it was present in the merest traces. Rubidium occurred in all specimens. In the transfused guinea-pig it reached the high value of 0·006 %, and it was nearly as high in the foetuses. In the blood rubidium was found in the red cells, and the destruction of these by the spleen may account for the richness of the spleen in this element. Silver occurred in one case only, and a trace of lithium was found in the spleen of K.T.

Kidney. In the guinea-pig and in foetus D the potassium lines were of the same intensity as in the organs already described, but in all the other specimens they were distinctly weaker. The magnesium line was strong in foetus D (0·2 %), but in all the other specimens represented 0·1 %. Traces of copper were present in every specimen, reaching the concentration of the weakest standard solution in four cases, two of which were foetal specimens. Manganese was absent from the kidneys of the two foetuses which died

Table IV. *Kidney.*

Patient	Copper	Manganese	Rubidium	Silver	Other elements
S.	Trace	Trace	0·0024	—	—
L.	0·002	Trace	Trace (faint)	—	—
F.M.	Trace	Trace	0·0024	—	—
K.T.	Trace	Trace	0·0024	—	—
A.B.	Trace	0·0008	0·003	—	—
G.E.	Trace	Trace	0·004	Trace	Lead (trace)
E.C.	Trace	Trace	0·003	Trace	—
Foetus D	0·002	Trace (faint)	0·005	—	—
Foetus (10)	0·002	—	—	—	Strontium (trace)
Foetus F	Trace	Trace	0·003	Trace	—
Foetus D (*a*)	Trace	—	0·0024	—	—
G.C.	Trace	0·0008	0·0024	—	—
G.P.	0·002	0·0008	0·004	—	—

in utero, but was present in small amount in all the other kidneys. Rubidium was absent from the kidney of the 10-weeks foetus and almost so from L. (myeloid leukaemia), but was present in significant amount in the other specimens. Traces of silver were found in three of the kidneys and lead in one. The kidney of the 10-weeks foetus was remarkable for containing a trace of strontium.

Table V. *Suprarenals.*

Patient	Copper	Manganese	Rubidium	Silver
S.	0·002	0·002	Trace	Trace
F.M.	Trace	Trace (faint)	Trace	Trace
K.T.	Trace	Trace (faint)	Trace	Trace
E.C.	Trace	Trace	0·003	Trace
Foetus D	Trace	Trace	0·006	—
Foetus F	Trace	Trace	0·0016	—
Foetus D (*a*)	0·002	Trace	0·004	—
Foetus L (2)	Trace	Trace	0·0016	—
G.P.	0·002	0·001	0·004	—

Suprarenals. Copper occurred throughout in small amount. Manganese was found in every case, but the variations were extraordinary (from a trace to 0·002 %). Rubidium occurred in small quantities in the post-natal specimens, while in the foetal ones measurable quantities were present. Silver was absent from the guinea-pig and foetal suprarenals, but occurred in traces in the others.

Urinary bladder. This organ was examined in two cases (A.B., G.E.) A trace of copper was present in each. Manganese occurred in A.B. as an obvious trace, while in G.E. the manganese line was only just visible. Rubidium was found in both specimens.

Intestinal canal. The stomach of F.M. and the duodenum of A.B. were examined. Traces of copper, manganese, and rubidium were found in both. A gastric ulcer removed by partial gastrectomy, compared with the adjacent normal stomach, showed a difference only in rubidium (normal 0·006 %, ulcer 0·0024 %), and in copper, in which element the ulcer was slightly richer. The Mn line in both cases was below the brightness of 0·0008 %. It is hoped to pursue this line of investigation in other cases.

Prostate. (M.L.) This organ contained traces of copper and silver, with 0·002 % of rubidium, but otherwise showed no unusual features.

Testis and epididymis. (M.L.) Both these organs contained traces of copper and the faintest traces of manganese. Rubidium was found in each (0·0016 %).

Uterus. (N.C., F.M.) The two specimens examined revealed no unusual features. Both contained traces of copper. Manganese was present as a trace in the uterus of F.M. but not of N.C. Rubidium was present in N.C. in a concentration of 0·0024 %, while in F.M. only a trace was found.

Placenta. The healthy 24-weeks placenta belonging to foetus D and that of foetus L (2) (myeloid leukaemia in the mother) were examined. Traces of copper and rubidium were present, but there was no manganese.

Ovary. (F.M., N.C., M.R.) The K lines were weak in F.M. Copper was present in small amount in all three, but the lines were distinctly stronger in the two patients who were below the age of the menopause. Manganese was absent, except for the merest trace in one of the ovaries of M.R. Rubidium gave a faint line (0·0016 %) in F.M. and slightly stronger lines (0·0024 %) in the other two. While there is obviously insufficient material for a generalisation, it is to be noted that the ovaries whose functional activity had not ceased were distinctly richer in potassium, copper, and rubidium, and a re-examination of the spectra shows that the same is also true of magnesium. There was, however, no difference between the spectra of the ovaries of the girl of 7 and of the adult of 27. The nature of any such changes in the mineral content of the ovaries which may set in after the menopause is under investigation.

Thyroid. (S, M.R., M.L., N.C. In the last case both the normal gland and a small adenoma were examined.) Copper was found in every case, but only in M.R. did it rise to a value of 0·002 %. Manganese was present in minute quantities in S. and in both specimens from N.C. but was absent from the other two. The rubidium lines were remarkably constant in all five specimens (0·002 %). Traces of silver were found in all the specimens. The normal gland was slightly richer in copper and the adenoma in manganese, but the differences were very small, and apart from these the spectra were identical. The thyroid of M.R. showed phosphorus, the lines of which were not visible in the other spectra.

Thymus. (Foetus D and foetus F.) The Mg line was stronger in the full-term foetus and the Cu lines were very much stronger (0·002 % as against a trace). The younger foetus, on the other hand, contained a trace of manganese, which was not present in the thymus of the elder. The Rb lines were strong in both (0·006 % in foetus D and 0·004 % in foetus F).

Tonsil. Four tonsils removed from children were examined. The spectra revealed practically no individual differences, but their general nature is interesting. Traces of copper were present in all but no manganese was found. The Rb lines were strong (0·003 %) and present in all. Every tonsil contained traces of silver.

Skin. The adult skin has not been examined, but the skin of foetus D contained 0·0024 % of rubidium, with traces of copper and manganese.

Heart. In all cases, pieces of heart muscle from the left ventricle were examined.

Table VI. *Heart.*

Patient	Copper	Manganese	Rubidium	Silver
S.	0·002	Trace	0·004	—
F.M.	0·002	Trace	0·004	—
K.T.	Trace	—	0·003	—
A.B.	Trace	—	0·004	—
G.E.	Trace	Trace	0·003	Trace
N.C.	Trace	Trace	0·003	Trace
E.C.	0·002	—	0·003	—
Foetus D	Trace	—	0·006	—
Foetus F	Trace	Trace	0·004	—
G.C.	Trace	—	0·002	—
R.M.	Trace	Trace	0·003	—
L (2)	0·002	—	0·0016	—
G.P.	0·002	Trace	0·006	—

Copper occurred throughout, but in only four human specimens was it more than a trace. Manganese was found in traces in six cases. Rubidium, on the other hand, gave lines that were remarkably constant in strength, corresponding to an average content of 0·0037 %. Silver was found in two of the specimens. No significant differences could be detected in the spectrum of E.C., death in this case being due to toxic myocarditis following diphtheria.

Aorta. (S., F.M., N.C.) As compared with N.C. (age 27) the Ca lines of S. (age 77) and F.M. (age 69) were markedly stronger, and, as is found in practically all the cases where the Ca lines are strong, the Mg line was much weaker. These changes are presumably the result of age. The merest traces of copper and rubidium were found in all three specimens. Manganese was only found in the aorta of S., and, as has been already mentioned, it gave a remarkably strong line (0·002 %). This aorta contained traces of silver and lead. Apart from any significance which may attach to the peculiar richness of this aorta in manganese, the presence of silver and lead in addition was presumably connected with the advanced degree of atheromatous change with calcification that was found.

Calcareous nodules from the case of S. were examined. These were found in the pericardium and on the mitral valve. The nodule on the valve was very small (0·168 g. dry weight) and it is possible that its general poorness in metals may be due to its small size making the traces present difficult of detection. The other nodule showed strong Cu lines (0·002 %), a very strong Mn line (0·002 %), and traces of silver and lead. It is evident from the changes found here and in the aorta that the process of calcification provides a nidus for the deposition of the heavy metals. Inasmuch as copper and manganese were present in such large amounts, one would have expected iron to be well represented, but this was not the case. The iron spectrum was absent from the smaller specimen, and only showed faint indications in the larger one.

Neither specimen contained rubidium. The K lines were distinctly weak. The Ca lines were naturally very strong, and the antagonism that appears to exist between calcium and magnesium was also found here, the Mg lines being very weak, indicating the presence of less than 0·02 % of this element.

Table VII. *Lungs.*

Patient	Copper	Manganese	Rubidium	Silver	Other elements
S.	Trace	Trace	0·0024	Trace	—
F.M.	Trace	Trace	0·0024	Trace	—
K.T.	Trace	—	0·0016	Trace	—
A.B.	Trace	Trace	0·0024	Trace	—
G.E.	Trace	—	0·0016	—	—
N.C.	Trace	Trace	0·0016	—	Lead (trace)
E.B.	0·002	Trace	0·0016	Trace	—
Foetus D	Trace	—	0·006	—	—
Foetus (10)	0·002	—	—	—	Strontium (trace)
P.	Trace	—	0·0024	—	—
Foetus F	0·002	Trace	0·0024	—	—
G.P.	Trace	Trace	0·006	—	—

Lungs. A noteworthy feature of the lung spectra is the strength of the iron spectrum. The difficulty of washing this tissue free of blood does not account for the feature entirely, as in the transfused guinea-pig the iron lines of the lung were stronger than those of any of the other organs with the exception of the spleen. In E.B. and in P. (both of which were cases of silicosis, E.B. being a very advanced one) the iron spectrum was very rich, in the case of E.B. being exceptionally so. Indeed, in E.B. the Fe lines were stronger than those obtained by burning the same weight of dried normal blood-clot, indicating that the silicosis was accompanied by a definite siderosis. The Mg line was faint in both these specimens. Copper was present in all the specimens, but only in E.B. and the two foetuses did it rise to 0·002 %. Manganese, in traces only, was found in 7 of the 12 lungs. Rubidium was found in 11 specimens without great variation in quantity except in foetus D and the transfused guinea-pig, both of which showed high values. Faint traces of silver were found in five specimens, and traces of lead and strontium each in one case.

Table VIII. *Muscle.*

Patient	Copper	Manganese	Rubidium	Silver
M.R.	Trace	—	0·006	Trace
M.L.	Trace	—	0·006	Trace
Foetus F (pectoral)	0·002	Trace	0·0024	—
Foetus F (diaphragm)	Trace	Trace	0·003	—
Foetus D (quadriceps)	Trace (faint)	—	0·004	—
G.C.	Trace	—	0·006	—
R.M.	Trace	—	0·006	—

Muscle. The Mg lines are weaker in the foetal specimens (0·1 %) than in the adult (0·2 %). Copper was found in all cases in small amounts, but the Cu content of the full-term foetus shows one feature which may be of importance. Pieces were taken from the pectoralis major and the diaphragm, and there is a distinct difference in the copper contents of the two, that of the diaphragm being the lower. The foetus was stillborn and had never

breathed, which suggests that the greater amount of copper in the pectoral muscle may be connected with its functional activity during the movements of intra-uterine life. In the adult muscles copper occurs in traces only, but all the organs are alike in showing a greater content of copper in the foetus than in the adult. Manganese was only found in the specimens from the full-term foetus. Rubidium occurred throughout, reaching a constant degree of concentration which is only equalled by the heart muscle and by the liver. Traces of silver were found in two of the specimens.

Calculi. Two calculi were examined, a single large cholesterol stone from the gall-bladder, and a small phosphatic calculus from the kidney.

The cholesterol stone contained the merest trace of magnesium, potassium, calcium, and iron, but a notable amount of copper was present (0·035 %). This figure agrees well with those found by Schonheimer and Oshima [1929] in their chemical analysis of six gall-stones, which varied between 0·016 % and 0·2 % Cu.

In the renal calculi the Ca lines were very strong, while both the K and Mg lines were weak. Manganese and rubidium were absent, but traces of silver and lead were found. There was only a faint indication of copper. The most remarkable feature is the presence of a well-marked Li line (0·0003 %). It is to be noted that the spectra of this and of the spleen of K.T. alone out of all those examined show a Li line. Some investigations now in progress, however, appear to show that this element is frequently found in the urine.

Cartilage. Five specimens of cartilage were examined. (Trachea from F.M., G.E.; rib cartilages from K.T., foetus D and foetus F.)

In G.E.—a man of 53—the K and Mg lines were weaker than in the others, while the Ca lines were stronger, presumably indicating a senile calcification. Copper was found in traces only in all the specimens except the full-term foetus (0·015 %). A trace of manganese was found in K.T. only. Rubidium occurred in all the specimens, the amount being smallest in G.E. while in the others it gave lines of approximately equal strength representing 0·003 %. A trace of lead was found in G.E. and the merest traces of iron were present in all five spectra.

Bone. (Parietal bone from K.T. and foetus D.) In both instances the weakness of the K and Mg lines and the strength of the Ca lines were the most prominent features of the spectra. Copper was just detectable, but manganese and rubidium were absent. The bone from K.T. contained a trace of lead.

Pituitary. (S.) Copper and manganese were both present in amounts of less than 0·002 % and 0·0008 % respectively. An infinitesimal trace of rubidium was found.

Brain. (Cerebral hemispheres S., K.T., foetus D., cerebellum K.T.)

The Mg lines were prominent throughout, and especially so in the case of the foetal brain (0·25 %). The Mg lines were stronger in the brain than in any other foetal organ. Copper was found in all the specimens, being strongest

in the foetal brain (0·0035 %). Manganese was absent from all, save for a very faint trace in the cerebrum of K.T. Rubidium was found in all cases. Traces only were present in the brain of K.T. but in the cases of S. and foetus D this element reached values of 0·004 and 0·003 respectively. Comparing the cerebrum with the cerebellum of K.T., the former, in addition to containing a trace of manganese, was definitely richer in both copper and magnesium.

Teeth. Seven teeth were examined, one of which was an incisor of the first dentition. In the adult teeth, no differences were found between the incisors, molars, and bicuspids. The spectra showed the following features.

(*a*) A very low magnesium content, this line being only just visible. As would be expected the Ca lines were very strong, and were stronger in the crown of the tooth than in the fang.

(*b*) The teeth were rich in sodium, but were low in potassium. The K lines indicated a content of less than 0·08 %, as against about 0·6 % for an organ like the liver.

(*c*) The iron spectrum was so faint as to be almost invisible, but the lines due to copper were quite clear. It will have been noticed that all the foetal organs and tissues appear to be much richer in copper than the adult ones, and this difference applies equally to the teeth. In the deciduous tooth, the copper amounted almost to 0·002 %, while in the adult teeth, the lines, though clearly visible, were much weaker. Manganese was not found.

Milk. Five specimens of human milk were taken, but unfortunately the stage of lactation they represented was not noted. There was, however, no specimen of colostrum. All the milks showed lines due to sodium, potassium, magnesium, and calcium. There was no marked variation in these metals except that three of the milks were lower in both potassium and magnesium than the other two. Iron was found in minute traces in all. Copper was present in all the specimens, in amounts of less than 0·002 % of the dry weight, except in one instance, where it just reached this value. Traces of rubidium occurred in two out of the five specimens (0·001 %), but manganese was not found in any.

Carcinoma. Only one specimen of carcinoma has been examined in this series—a squamous-celled carcinoma of the oesophagus. Traces of copper and rubidium were found, but no manganese. The spectrum apart from these features, showed nothing unusual.

Blood. The results of the examination of the blood will be found in Table IX. In the case of blood and serum the examination was carried out on 0·1 cc., and in the case of the solid clot, on 0·05 g. of the dried material. No attempt has yet been made to examine the plasma as such, as it was feared that by the use of either vaselined tubes or citrate to prevent clotting, impurities might be introduced. The method adopted was to allow the blood to clot and the serum to separate. This was removed by pipette and centrifuged. The clot was then washed rapidly in distilled water and placed in the hot steam-oven.

In a few minutes a pool of serum was expressed from the clot. This was removed, the clot broken up and again rapidly washed in distilled water, before being finally dried. The method does not, of course, effect a complete separation of the clot from the serum, but it was thought best at this stage to employ it, rather than introduce the use of chemicals.

Table IX. *Blood.*

	Disease	Age	Sex	Copper	Manganese	Rubidium	Silver
1.	Anaemia of pregnancy	40	F.	0·002	—	—	Trace
2.	37 weeks pregnancy	—	F.	Trace	—	Trace	—
3.	Full-term ,,	—	F.	Trace	Trace	Trace	—
4.	36 weeks ,,	—	F.	Trace	—	Trace	Trace
5.	38 weeks ,,	—	F.	Trace	Trace	Trace	—
6.	12 weeks ,,	—	F.	Trace	Trace	Trace	—
7.	22 weeks ,,	—	F.	Trace	Trace	Trace	Trace
8.	Diffuse hyperplastic sclerosis	40	F.	Trace	—	—	—
9.	Myeloid leukaemia	?	F.	Trace	—	—	—
10.	Myeloid leukaemia. Clot	30	M.	Trace	—	0·006	—
11.	,, Serum			Trace +	Trace	—	—
12.	Foetus D. Heart blood (Dried whole blood)	—	—	Trace	—	0·004	—
13.	Foetus F. ,, (Dried whole blood)	—	—	0·005	—	0·004	—
14.	Foetus D (*a*). Liver blood (Dried whole blood)	—	—	0·005	—	0·002	—
15.	22 weeks pregnancy (clot)	—	F.	Trace	—	0·003	Trace
16.	,, (serum)	—	F.	Trace	Trace	—	—
17.	Paget's disease of bone (0·1 cc. blood)	25	M.	Trace	Trace	0·0016	—
18.	,, (serum)			Trace +	Trace	—	—
19.	,, (clot)			Trace	—	0·003	—
20.	Myxoedema	35	M.	Trace	—	Trace	Trace
21.	Carcinoma of ovary	28	F.	0·002	Trace	Trace	—
22.	Chlorosis	15	F.	0·002	Trace	Trace	—
23.	Tabes dorsalis	38	F.	Trace (faint)	Trace	—	—
24.	Cirrhosis of liver	63	F.	Trace (faint)	—	—	—
25.	Perinephric abscess	35	M.	Trace (faint)	—	—	—
26.	Pernicious anaemia	60	M.	Trace (faint)	Trace	—	—
27.	Polycystic kidney	36	M.	Trace (faint)	Trace	—	—
28.	Landry's paralysis	24	M.	Trace (faint)	Trace	—	—

The iron spectrum was strong in all the specimens of blood, with individual variations depending on the presence of anaemia. A very faint trace of iron was also seen in the centrifuged serum. Sodium appeared to be present in the serum in greater amount than in the clot. Potassium, on the other hand, was found chiefly in the red cells, the potassium spectrum of the serum being very weak. Copper occurred in all the specimens. It reached a strength that could be compared with the weakest standard solution in two of the three foetal cases, but only one of the adult bloods was sufficiently rich in copper to do this. It happened that this—a case of severe anaemia of pregnancy—was the first blood to be spectrographed, and the finding of an unusual amount of copper led to six other specimens of blood from cases of normal pregnancy being examined. The fact that the foetal tissues are so rich in copper, which must be supplied by the mother, gave rise to the expectation that pregnant blood might, in general, be characterised by such a richness in copper as was found in this case. The copper contents of the six pregnant bloods were higher than in the other adult bloods, but nowhere as high as in the anaemia of pregnancy. Evidently the large demands made by the foetus for copper cause

this slight increase in the level of copper in the blood during pregnancy. The association in one case of a severe anaemia with an unusually high level of copper suggests that for some unknown reason there was an unusual mobilisation of the copper reserves of the mother, and our present knowledge of the relation between copper and the formation of haemoglobin suggests that it was the depletion of this which led to the anaemia.

In all the specimens examined, the copper appeared to be equally distributed through the serum and the clot. Manganese was found in eight of the specimens. In the three cases where clot and serum from the same blood were examined, the manganese was invariably found in the serum, but not in the clot. Rubidium, on the other hand, showed the reverse distribution, being found in the clot but not in the serum.

Traces of silver were found in the blood of five patients. It only happened to occur once in the cases where both clot and serum were examined, and then it was found in the clot, but not in the serum.

The bloods from myeloid leukaemia, myxoedema and diffuse hyperplastic sclerosis were not distinguished by any peculiar features. Eighteen sera which had been tested for the Wassermann reaction were examined, but no differences were noted between the positive and negative sera.

Lymph-gland. A gland from L. (lymphatic leukaemia) showed traces of copper and rubidium, but no other features of note. A partially calcified tuberculous gland from the mesentery of another patient, showed a trace of manganese in addition.

Bone-marrow. Marrow from the middle of the shaft of the femur of a man of 25, who died of miliary tuberculosis, was examined. The spectrum is poor in potassium, and neither manganese nor rubidium is present. Copper amounted to slightly less than 0·002 %.

Discussion.

The detailed description of the various human tissues shows that in addition to sodium, potassium, calcium, magnesium and iron, copper is a universal constituent of protoplasm, rubidium is almost universal, while manganese and silver, in addition to an apparently irregular distribution, are also constant constituents of certain organs. Each of these metals will be discussed separately.

Copper.

Every specimen taken from living tissue contains copper, though in a tissue such as bone the amount present is very small. The presence of copper in the tissues of plants and animals has been recognised for a long time, its occurrence being regarded as of either accidental or toxic nature, and it is only in recent years that the possibility of its being an element essential to life has had to be considered. The well-known work of Waddell *et al.* [1928, 1929], demonstrating the essential rôle played by copper in the formation of

haemoglobin, is in keeping with the trend of other recent work which has demonstrated the widespread distribution of copper in living tissues [Lindow, Elvehjem and Peterson, 1929]. In a recent paper, Fox and Ramage [1931] found copper to be present in every one of 146 specimens of tissues removed from different species of invertebrates. In the present investigation, copper has been found in every one of 195 specimens of tissue removed from 23 human beings. The constant presence of copper in over 500 tissues from all parts of the animal and vegetable kingdoms, together with the evidence of McHargue [1925] and Maquenne and Demoussy [1920] as to its distribution in the plant world, leads to the conclusion that copper is a universal constituent of protoplasm. That it is also an essential constituent is rendered likely by its known importance in relation to haemoglobin formation in mammals and its occurrence in haemocyanin.

There are certain aspects of the distribution of copper in human tissues which merit separate attention.

(1) It is obvious that copper is of great importance in the functions of the liver during foetal life. The copper values in the five foetuses examined are between seven and ten times in excess of the adult percentage. This is in agreement with the finding of McHargue [1925] who in the liver of an ox found 50 mg. of copper per kg. of dry tissue, while in that of a calf born dead the figure was 908 mg. of copper per kg. It is probable that part at any rate of the peculiar importance of copper for the foetal liver may be connected with its blood-forming functions in this stage of life.

(2) The other foetal organs also contain a greater amount of copper than the corresponding adult tissues, though the difference is far less than in the case of the liver. This has been found by other workers. Thus Bodansky [1921] found that the copper content of the foetal brain is greater than that of the adult. Copper presumably plays an important part in the phenomena of growth. This seems to be the most feasible explanation of the difference in the copper contents of an incisor of the first dentition and of various teeth taken from adults. In view of the knowledge that is being accumulated by the work of Mellanby [1931] and others on the relation between an adequate vitamin supply and the formation of sound teeth, and the suggestion of McHargue [1925] that copper may play some part in the formation of certain of the vitamins, the relations that may exist between copper and the growth of the teeth merit further investigation.

(3) It is obvious that to supply the great demands of the foetus for copper, inroads must be made on the maternal store of this metal, unless a sufficient amount to meet the foetal demands is present in the food during pregnancy. The drain of copper from the mother does not cease after birth, for the examination of five specimens of human milk showed copper to be present in all. That it would be possible to take a diet deficient in copper is indicated by the investigation of Lindow, Elvehjem and Peterson [1929]. It therefore seems possible that the chronic secondary anaemia and general debility found

in some cases of pregnancy may be connected with a dietary deficiency of copper. The whole question of the metabolism of copper during pregnancy seems a promising field for investigation. Its probable importance is further suggested by the solitary observation that, of the pregnant bloods that were examined, the greatest amount of copper occurred in a case of severe secondary anaemia, which may have been due to some unusual demand of the foetus for copper leading to an abnormal mobilisation of the maternal store of this metal.

(4) Of the various diseases that were investigated, the only ones associated with marked abnormalities of copper content were those represented by R.M. (chronic interstitial nephritis) and J. (atrophic cirrhosis of the liver). In the absence of more extended knowledge, the meaning of the large amount (0·02 %) of copper in the livers of these cases must be left an open question.

(5) The presence of copper in bile (and in a gall-stone) suggests that this is a channel of excretion for the metal. In the case of two sheep that were examined, the copper lines were visible on burning 0·1 cc. of bile, while the average content of their gall-bladders (dry) was 0·005 %. The high figures also obtained in the human gall-bladders suggest that the body attempts to conserve its supply of copper by reabsorption through the wall of the gall-bladder, and this probably occurs through the intestine as well.

Manganese.

(1) Manganese is especially found in the liver, pancreas, kidney and suprarenal, and to a less extent in the heart and lung. Its distribution through the other tissues is irregular. This differs in several respects from the findings of Zbinden [1930], who obtained manganese only in the liver and kidney. Our results agree in showing that the liver contains the greatest amount of this metal.

(2) The high content in manganese of the tissues from the transfused guinea-pig is noteworthy.

(3) Of the human specimens, the greatest individual amounts are found in the tissues of S. All the organs contain normal amounts with the exception of a calcified nodule from the heart, the suprarenals, and the aorta. This man died from cerebral haemorrhage at the age of 77, and the unusually high amounts of manganese in the aorta and suprarenals especially, raises the interesting possibility that the development of arteriosclerosis may be connected with the metabolism of manganese.

(4) The examination of the liver of J. lends no support to the thesis that cirrhosis of the liver may be due to chronic manganese poisoning.

(5) Manganese was found in 14 out of 28 specimens of blood, its presence being confined to the serum.

Rubidium.

Rubidium lines were found in 16 of the 28 bloods examined. Rubidium is present in the cells, and not in the serum.

(1) Rubidium has a very much wider distribution than manganese in the tissues. This may be due to its greater diffusibility.

(2) The distribution of rubidium is, in general, the opposite of that of manganese. While the latter is chiefly found in the glandular organs such as the liver and pancreas, being present in traces in 7 only of the 12 hearts and in 1 only of the 6 specimens of muscle, rubidium reaches its greatest concentrations in the heart and striated muscle.

(3) The only tissue from which rubidium is absent in the adult is bone.

(4) The only individual to be without rubidium was the 10-weeks foetus. It is impossible to say whether this is a chance result, or due to the early age of the foetus, or to be associated with other mineral abnormalities that were present.

(5) In general, this element appears to be associated with potassium in its distribution through the tissues, the strength of the lines due to the two metals tending to vary harmoniously. It must be borne in mind, however, that the brightness of the lines of these two elements may not be entirely independent quantities, since Gooch and Phinney [1892] by eye-observations on low temperature flames have shown that the presence of potassium salts in minute quantities will materially increase the brightness of the rubidium spectrum, while the reverse occurs when the potassium is present in larger amount.

(6) Rubidium has been found in this investigation to be almost universally present in tissues. The fact that Zbinden [1930] makes no reference to it in the course of his work is probably due to the difference in technique—since he uses an arc spectrum while we employ a flame spectrum—and in a paper on milk he states that rubidium "ne donne pas de raies spectrales assez sensibles entre 2500 and 3500 Å."

The trend of previous work on the physiological behaviour of rubidium is to show that the metal can partially or completely replace potassium in Ringer's solution. Ringer himself [1883] noted this action, and it has been confirmed by Zwaardemaker [1919] and others. Loeb [1920] finds that rubidium chloride is able entirely to replace potassium chloride in the solution required for the development of the eggs of the sea-urchin into swimming blastulae. Mitchell *et al.* [1921] by perfusing frogs with Ringer's solution in which the potassium chloride was replaced by rubidium chloride found that the rubidium was taken up by muscles which had been kept in activity, but was not taken up by resting muscle. In feeding rats with rubidium, they found that the bulk of this metal was stored in the muscles, which is in agreement with the findings of the present paper. Mendel and Closson [1906], after the injection of rubidium salts into rabbits and cats, came to the same conclusion, stating that "muscles form the chief storehouse for the temporary retention of rubidium, in which they surpass the liver." That the inter-availability of potassium and rubidium in this way is not the whole story is shown by further experiments of Mitchell and Wilson who fed young white

rats on a purified synthetic diet in which the salts of potassium were replaced by those of rubidium. After 10 to 15 days the rats showed marked derangement, with irritability and tremblings, developing later into tetanic spasms, and death within 48 hours. In all these experiments it was also found that caesium behaved like rubidium in all respects. The physiological behaviour of both caesium and rubidium so far as it is known, appears to be identical, yet caesium has not been found in any of the present series of tissues, or in a series of examinations made by Fox and Ramage [1931] on animals of widely different genera. It is difficult to say whether this is due to some unknown difference between the physiological properties of the two elements or to the fact that caesium, though as widely distributed in nature as rubidium, is not present in such great amounts. In sea-water the proportion of caesium to rubidium is about 1:200. It is possible that it may be present in living tissues, but in such small amounts that very much larger quantities would have to be burnt than were employed here, in order to demonstrate its presence. The presence of rubidium in tissues has a further interest in that it also occurs in sea-water, the relationships between the mineral composition of blood-plasma and sea-water being a well-known phenomenon. Rubidium may prove to be of further importance in that it is a faintly radio-active element. In this respect it resembles potassium. Zwaardemaker [1919] finds that rubidium can replace potassium in Ringer's solution in proportion to their relative radio-active powers. Roffo and Landaburu [1925] find that the radio-activity of organs injected with rubidium chloride is variable, but that tumour tissue always shows a higher radio-activity than normal tissue, presumably owing to the fact that tumour tissue absorbs more rubidium.

Silver.

Silver is present in considerably less quantity than the preceding metals, never rising to 0·001 % of the dry weight. It was found in every specimen of thyroid and tonsil examined (8 in all). It was found in the other organs in the following percentage of cases: liver, 47 %; suprarenal, 44 %; lung, 36 %; muscle, 33 %; pancreas, 30 %; kidney, 23 %; heart, 14 %; spleen, 8 %; isolated instances occurred in the aorta and a calcareous nodule. It was absent from the rest of the tissues. In blood, traces of silver were found in 5 out of 28 cases. Its presence does not appear to be connected with age and is not universal, since it is absent from all the tissues examined from foetus D and from the case of R.M., though in neither of these were either the tonsils or thyroid examined. None of the guinea-pig specimens contained silver. Whether this element has any physiological function, or whether its presence is accidental, its irregular distribution being due to selective absorption by the different tissues, is uncertain. Zbinden [1930] using the arc spectrum has found silver in every human tissue he has examined, including blood; thyroid being especially rich in silver, with which these investigations are in agreement.

Lead.

This occurred only spasmodically. It was found in the liver, kidney and cartilage of G.E., the aorta and a calcareous nodule of S., the bone of K.T., the renal calculus and in the lung of N.C. This varied and irregular distribution strongly suggests that the metal is present accidentally.

Strontium.

This metal was found in the kidney of the guinea-pig, and in the liver and lung of the 10-weeks foetus. This scanty amount does not suggest that the metal has any physiological functions, and it has not been described by Zbinden [1930] as being found by his spectroscopic method. Some unpublished observations of ours, however, indicate that strontium is probably a constant constituent of the retina. It is curious that in the present investigation the 10-weeks foetus should provide two out of the three instances where this element occurs. The whole mineral content of this foetus appeared to be abnormal, being deficient in potassium, devoid of rubidium, and containing strontium and a high proportion of calcium. It would be interesting to enquire whether such early foetal death may, in other cases, be associated with abnormalities of their mineral constitution, or if the composition found is normal for that age.

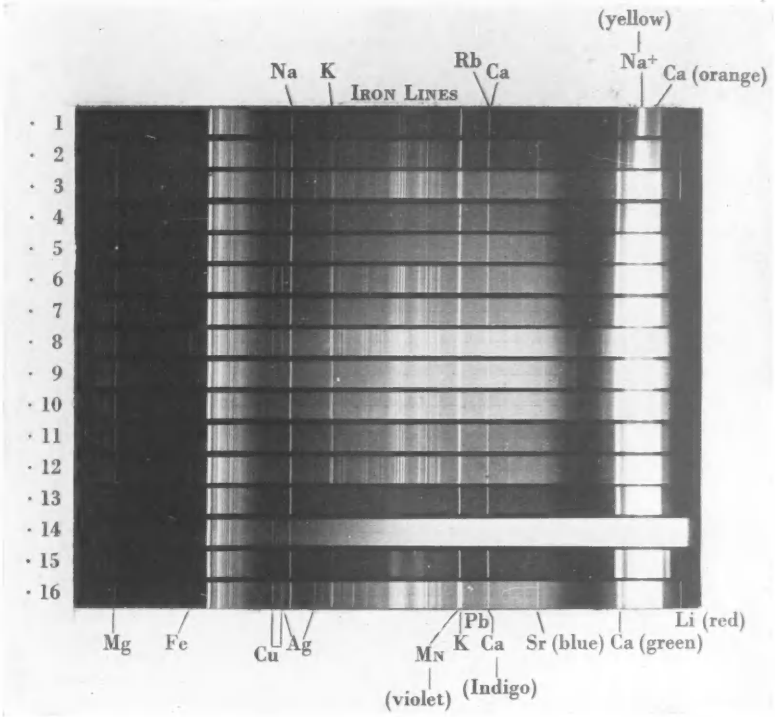
Lithium.

In view of its wide distribution through the animal kingdom, it was expected that this element would be of frequent occurrence, and its rarity came as a surprise. A lithium line was only found in two instances—a renal calculus and the spleen of K.T. Further work is required, since Hermann [quoted by Zbinden, 1930] states that it occurs in the foetus, and in the adult is concentrated particularly in the lungs, while Keilholz [1921] actually gives a table of values for the lithium content of the various organs. Zbinden [1930], however, makes no reference to lithium. In the course of some further work, we have found lithium to occur in human urine. It is possible that the differences which have been found are related to local variations in the composition of the soil or the water supply.

SUMMARY.

1. Copper is a universal, and is probably an essential, constituent of living tissue. It occurs in greater concentration in foetal tissues than in adult, and reaches its maximum amount in the foetal liver, where the percentage of copper may be as much as ten times in excess of that present in the maternal liver.

2. Manganese occurs spasmodically in many tissues, but is chiefly concentrated in the liver, pancreas, suprarenal and kidney. It is present in bile, foetal bile containing a high proportion. In the blood it appears to be confined to the serum.



No. 1. 0·02 cc. standard solution.
2. 0·05 cc. standard solution.
3. 0·1 cc. standard solution.
4. Pancreas. Foetus D.
5. Gall-bladder and bile. Foetus D.
6. Liver. Foetus D.
7. Suprarenal. Foetus D.
8. Blood from liver. Foetus D.
9. Kidney. Foetus D.
10. Spleen. Foetus D.
11. Quadriceps muscle. Foetus D.
12. Blood (clot). Myeloid leukaemia.
13. Blood (serum). Myeloid leukaemia.
14. Gall-stone.
15. Renal calculus.
16. 0·2 cc. standard solution.

3. Rubidium is almost as widely distributed as copper and occurs in greatest concentration in the heart and in striated muscle.

4. Silver is found to a less extent than the preceding metals, but appears to be a normal constituent of the thyroid and the tonsil.

5. Lead, strontium and lithium were only found to occur spasmodically.

It is hoped to pursue the various points that have been raised in the course of this investigation.

We wish to express our thanks to all those who have helped in the supply of tissues—to Dr S. C. Dyke, Director of the Pathological Department, both for the supply of material and for allowing the use of the Laboratory for part of the work, to Dr W. Stewart, the assistant Pathologist, to Mr Maslen Jones and Dr Wilkes of the Maternity Department for their help in obtaining foetal tissues and to the Royal Society Government Grant Committee for grants for the purchase of spectrographic apparatus.

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CLXXIV. BLOOD-PHOSPHORUS IN FAT ABSORPTION.

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(*Received August 13th, 1931.*)

The view held that the phosphatides are concerned in the transport of fat for utilisation or deposition in the tissues seems to have found rather a general acceptance. To a great extent this view is experimentally based on the observation that the phosphatide content of blood is very often increased on occasions when an increased fat transport with the blood is presumably taking place, *e.g.* after fat ingestion. This phenomenon has been studied particularly by Bloor [1915, 1916], but has also been observed by many other investigators, usually in experiments on dogs[1].

According to Bloor [1916] most of the increase of the phosphatides takes place in the blood-corpuscles, in which this author found at the same time an augmented content of glycerides. On the strength of these observations Bloor advanced the interesting and much cited hypothesis that the fatty acids absorbed from the intestine are converted into phosphatides in the corpuscles and mainly as such are removed from the blood. Noticeably little additional work has, however, been done in order to test the correctness of this hypothesis. Knudson [1917] confirmed the observation of phosphatide increment in the corpuscles after fat ingestion. On the other hand Bloor [1920] himself in a later paper mentions that "further unreported work in this laboratory has not, however, entirely borne out these findings, since certain dogs have been found, in which the increase of lipoid phosphorus took place in the plasma and not in the corpuscles." Further Iwatsuru [1926] found no such increase (in experiments with rabbits). Under these conditions I have thought it desirable to submit the question of the alimentary phosphatide increase in the corpuscles and in plasma to renewed investigation. For a further trial of the hypothesis in question I have, concurrently with the determination of lipoid P, also determined the non-lipoid P of plasma and corpuscles. This was done with the idea that if the phosphatides added to the blood during fat absorption are formed in the blood itself, either in corpuscles or in plasma,

[1] It is noteworthy that no alimentary increase of blood phosphatides has been found in man [Hiller, Linder, Lundsgaard and Van Slyke, 1924; McClure and Huntzinger, 1928; Page, Pasternack and Burt, 1930]. This discrepancy is not unlikely to be explained by the fact that the human subjects tested in this respect have received, in spite of the ingestion of about 100 g. of fat, a much smaller quantity of fat in relation to their body weight than the dogs.

it might reasonably be expected that the non-lipoid P of the blood should show a simultaneous decrease.

Methods.

The investigations were performed on dogs which were starved for 24 hours before the test-meal. Between the experiments the dogs were fed on a mixed diet, for the main part consisting of meat. Blood (about 15 cc. each time) was drawn from a cutaneous vein on the hind leg just before the fat ingestion and 2, 4, 8, 12 and generally also 24 hours afterwards. The test meal consisted of olive oil in an amount of about 5 cc. per kg. body weight (usually 100–120 cc. in all) and of 100–200 g. of lean meat. The oil was given by stomach tube or simply by pouring it directly into the pouch produced in the mouth of the dog, by pulling the lips away from the jaws on one side. In Exps. 5, 6 and 7 the animals were given 100 g. of glucose in water (+ 0·25 g. of tannic acid) 18 and 12 hours before the fat meal (*i.e.* 200 g. of glucose in all). In Exp. 6 no meat was given. The blood was transferred to a flask containing solid sodium oxalate in a quantity of about 0·2 % of the blood taken. After the oxalate had been mixed up with the blood, this was centrifuged for 30 minutes at a rate of 2700 r.p.m. and the plasma cautiously drawn off. The leucocyte layer and the uppermost layer of the red corpuscles were removed and the rest of the erythrocytes taken for analysis.

For the phosphatide determination 2 cc. of plasma were dropped slowly into 40 cc. of a mixture of 3 parts of alcohol and 1 part of ether which was kept rapidly rotating. The mixture was heated to boiling in a water-bath and thereafter cooled to room temperature. The entire fluid was then filtered into a 100 cc. Erlenmeyer flask, two washings with 10 cc. of the alcohol-ether mixture being employed. The filtrates were evaporated to dryness on a water-bath, the residue incinerated with sulphuric and nitric acids and the phosphorus determined titrimetrically according to the modified Neumann method, described by Widmark and Vahlquist [1931].

To a weighed quantity (about 1·5 g.) of corpuscles was added an approximately equal amount of water and the mixture again weighed. The fluid was then shaken vigorously and allowed to stand for a few minutes. The haemolysed corpuscles were poured drop by drop into the alcohol-ether mixture and the amount poured in was determined by weighing before and after. For the rest the determination was performed in the same way as with the plasma.

In plasma the so-called acid-soluble P is practically identical with the non-lipoid-P. The acid-soluble P was determined as follows. 4 cc. (in some cases only 2 cc.) of plasma were added drop by drop to an Erlenmeyer flask containing ten times the amount of saturated picric acid in 0·25 % hydrochloric acid. After standing for 1 hour the mixture was filtered and an aliquot part (usually 30 cc. out of 44 cc.) was evaporated, incinerated and the phosphorus determined according to the Widmark-Vahlquist method.

In the corpuscles the non-lipoid P was determined as the difference

between total and lipoid P. For the determination of the total P of the corpuscles quantities of about 0·75 g. were weighed, thereafter incinerated and analysed for P as above.

The experimental confirmation of the accuracy of the methods used for the determination of lipoid P and acid-soluble P of plasma was given by Widmark and Vahlquist [1931]. For the quantities of plasma used in the present work the error does not as a rule exceed ± 0·05 mg./100 cc. With regard to the determination in the corpuscles the accuracy becomes somewhat less. According to our experience the error of the determination of lipoid P and of total P in the corpuscles seldom exceeds ± 0·2 mg./100 cc. and ± 1 mg./100 cc. respectively.

Table I. *Experiments with fat ingestion.*

		Plasma		Corpuscles		
Exp. No. Date Exp. animal	Hours after fat ingestion	Phos-phatide P mg./100 cc.	Acid-soluble P mg./100 cc.	Phos-phatide P mg./100 cc.	Non-lipoid P mg./100 cc.	Turbidity of plasma
I	0	—	—	14·2	48·6	0
20. viii. 1930	2	16·1	5·3	14·3	51·2	+ +
Dog 1. ♂	4	17·4	5·1	14·9	49·2	+ + +
	8	16·8	5·1	14·2	49·9	+(+)
	12	16·9	4·6	14·1	50·0	+
	24	14·2	3·1	14·3	47·1	0
II	0	15·3	3·9	16·6	53·6	0
27. viii. 1930	2	16·1	4·7	16·2	54·1	+ +(+)
Dog 1. ♂	4	17·4	5·4	17·0	50·8	+ + +
	8	16·6	4·7	16·8	51·8	+ +
	12	15·6	5·4	16·3	54·1	+
	24	14·5	3·3	16·5	51·7	0
III	0	14·7	4·5	16·7	48·8	0
3. x. 1930	2	14·4	5·0	16·5	51·0	(+)
Dog 2. ♂	4	16·9	6·2	16·0	48·0	+
	8	15·8	6·1	17·0	48·3	+(+)
	12	15·0	5·1	16·4	50·1	0
IV	0	12·3	3·3	14·6	—	0
26. x. 1930	2	12·0	3·3	13·8	—	+ +
Dog 3. ♀	4	14·4	4·0	15·0	—	+ + +
	8	13·3	3·3	14·5	—	+
	12	11·9	4·1	14·4	—	(+)
	24	11·6	3·7	14·2	—	0
V	0	12·2	3·6	15·5	51·0	0
12. x. 1930	2	12·8	3·4	15·2	49·0	+ +
Dog 1. ♂	4	14·4	3·8	15·3	50·8	+ + +
	8	15·4	3·8	14·6	49·4	+ + +
	12	15·5	4·4	14·7	52·7	+ +
	24	14·8	3·4	14·7	52·2	(+)
VI	0	9·7	3·7	14·4	—	0
17. xii. 1930	2	10·0	4·0	14·3	—	+(+)
Dog 2. ♂	4	11·1	—	14·5	—	+(+)
	8	11·4	3·7	14·1	—	(+)
	12	11·2	4·6	14·4	—	0
VII	0	9·7	4·3	14·7	—	0
20. xii. 1930	2	9·8	4·9	—	—	+
Dog 2. ♂	4	11·5	4·6	14·2	—	+ +(+)
	8	10·3	4·7	14·5	—	(+)
	12	9·9	4·9	14·4	—	0
	24	9·9	4·4	14·0	—	0

+ cloudy, + + slightly milky, + + + milky

Experimental results.

The analytical data are comprised in Tables I and II. In all experiments with fat ingestion (Table I) there is a clear increase of the plasma phosphatides, varying between 14 and 26 % of the fasting values. In five of the experiments the maximum is reached after 4 hours, in one experiment after 8 hours, and in another experiment after 12 hours. In the last two cases the animals were given glucose before the fat meal. On the contrary the phosphatides of the corpuscles show only small and quite irregular changes. As to the acid-soluble P increases were found in all the experiments (in No. 1, the fasting value was lost), in some cases small indeed, but certainly exceeding the experimental error. In Exps. 2 and 3 the rise and fall are regular with a maximum 4 hours after fat ingestion. In the rest of the experiments the changes are more irregular but in no instance is there a significant fall below the fasting values. The level of the non-lipoid P of the corpuscles is not moved definitely in any direction. Small variations occur but in quite an irregular way. In all experiments the plasma became markedly lipaemic, the maximal milkiness appearing as a rule after 4 hours. In the three control experiments (XI–XIII, Table II) in which lean meat was given but no oil the phosphatides of the plasma remained constant but the acid-soluble P of the plasma showed a rise of about the same magnitude as in the experiments with fat ingestion. In the three control experiments (VIII–X, Table II), with no food supply the plasma phosphatides as well as the acid-soluble P were unchanged.

Table II. *Control experiments.*

Exp. No. Date Exp. animal	Hours	Plasma		Corpuscles
		Phosphatide P mg./100 cc.	Acid-soluble P mg./100 cc.	Phosphatide P mg./100 cc.
VIII	0	11·7	4·5	—
24. i. 1931. Dog 1. ♂	4	11·6	4·3	—
IX	0	11·4	3·4	—
15. ii. 1931. Dog 1. ♂	4	11·3	3·5	—
X	0	9·9	3·5	—
15. ii. 1931. Dog 2. ♂	4	10·0	3·6	—
XI	0	15·5	3·5	14·1
14. viii. 1930. Dog 3. ♀	5	15·2	5·2	13·6
XII	0	11·5	3·7	—
24. i. 1931. Dog 2. ♂	4	11·5	4·4	—
XIII	0	11·7	4·2	—
30. i. 1931. Dog 1. ♂	4	11·7	5·0	—

Discussion.

My experiments were arranged in much the same way as Bloor's experiments in which this author found a marked phosphatide increase in the corpuscles. Among the obvious differences entailed in the experiments, however, none can to my mind be presumably held responsible for the marked discrepancy found in the results. My dogs (with one exception) were given

some meat together with the oil, whereas Bloor's animals had oil only. In Exp. 6 in which I gave no meat the outcome is, however, quite the same as in the rest. I made my determinations directly on the corpuscles, whereas Bloor obtained his corpuscle values as differences between whole blood and plasma, guided by haematocrit determinations. Bloor's corpuscle values, therefore, contrary to mine, include the leucocyte phosphatides. Yet, not even a considerable digestive leucocytosis, in spite of the relative richness of the leucocytes in phosphatides [Boyd, 1931] could produce the difference. The facts that I drew the blood from a vein of a hind leg, whereas Bloor used blood from the jugular vein and that different analytical methods were employed cannot reasonably explain the different results. There are certain reasons for believing that the diet during the period preceding the experiments might have been of some influence. According to Wendt [1928] and to Rony and Ching [1930] the fatty acid increase in plasma is much higher in animals which have fasted for some days previous to the fat meal, than in animals fed in the usual way. Bang [1918], Rony and Ching [1930] and others have found that ingestion of carbohydrates together with fat tends to repress the alimentary lipaemia. On the other hand, our experiments in which glucose was given before the fat meal show practically the same results with regard to phosphatides and non-lipoid P as the rest. In two of the glucose experiments the phosphatide increase in plasma appears somewhat protracted, but in the third the course of the phosphatide level was quite the same as in the experiments without glucose. Bloor does not mention anything about the diet used between the experiments. My animals received, as stated above, a mixed diet consisting mainly of meat. It remains to state that at present it is impossible to explain why a fat meal should be followed by a phosphatide increase in the corpuscles in some dogs and not in others.

After having found that in haemorrhagic lipaemia the phosphatide increase in the corpuscles is inconsiderable as compared with that in plasma, Bloor suggests that the absence of the rise in the corpuscles here and in certain cases of alimentary lipaemia may be due to the rapid passage of lecithin from the corpuscles to the plasma. Yet if this assumption were right, the new formation of lecithin in the corpuscles would be expected to cause a decrease of the non-lipoid P of the corpuscles and eventually of the plasma, especially when the new formation of lecithin had been going on for some time, that is towards the end of fat absorption. In our experiments, however, the non-lipoid P shows in no instance the least tendency to fall. On the contrary, in the plasma the non-lipoid P shows some increase. (This increase could possibly be ascribed to the meat given, as it was only in the control experiments with meat that a similar rise was found. As in Exp. 6, where only oil was given and no meat, an increase was also found, the fat may be at least partly responsible for the rise in the rest of the experiments.)

My results are thus clearly against the view of a phosphatide formation within the corpuscles during fat absorption. They cannot, however, be said

definitely to disprove it. To settle this question qualitative investigations of the corpuscle phosphatides during fat absorption will no doubt also be necessary.

SUMMARY.

1. In experiments on dogs the phosphatides of plasma showed after fat ingestion a regular increase, whereas the phosphatides of the corpuscles remained practically unchanged.

2. The non-lipoid P of plasma showed a small increase during fat absorption, the non-lipoid P of the corpuscles being unaltered.

3. The results argue against the assumption that the fat absorbed from the intestine is transformed into phosphatides of the red corpuscles of the blood.

The writer wishes to thank Prof. G. Blix for suggestions and advice in the work.

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CLXXV. THE DETERMINATION OF TRYPTOPHAN AND TYROSINE.

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The colorimetric estimation of tryptophan and tyrosine, published by Folin and Ciocalteu [1927], has been used lately by several workers. Marston [1928] determined the amounts of these amino-acids in keratin from sheep's wool and considered the determinations to be satisfactory. Hanke [1928] found that the method could not be used for the estimation of tyrosine in the presence of cystine. He noted further, that a crude protein hydrolysate, either acid or alkaline, appeared to contain something other than tyrosine and tryptophan which interferes with the determination of these amino-acids. McFarlane and Fulmer [1930], using the method for the estimation of these amino-acids in some food proteins, experienced similar difficulties. More recently, Carpenter [1931] has used the method in determining the molecular weight of caseinogen. He does not appear to have recognised any difficulty except that he observes that only 81·3 % of added tryptophan was recovered. He estimated tryptophan, not only colorimetrically, but also by bromination.

This latter modification has been subjected to an investigation in this laboratory for the past two years which seems to demonstrate some important difficulties which are likely to be encountered in carrying out the procedure. The work was undertaken as part of an investigation into certain discrepancies in the methods of protein analysis, particularly with reference to the Van Slyke method. It was found that the decomposition products of tryptophan are likely to become involved, so that it was desirable to use a rapid and simple method of estimating tryptophan. As will be shown, this object has not altogether been achieved, but when using this procedure it is necessary to take into account the presence of histidine. The method appears to offer as good an estimation of histidine as it does of tryptophan. The main part of the investigation will form the subject of a further communication, but in view of a somewhat general adoption of the method of estimating tryptophan and tyrosine, it is thought that publication of these experiments, at this time, may be of interest.

Experimental.

Plimmer and his associates [1913, 1924] have shown that bromine may be absorbed by tryptophan, tyrosine, histidine, and cystine. Homer [1915] obtained slightly higher results for tryptophan.

Two cc. of a 1 % solution of each amino-acid (except tyrosine) were used for each determination, and were brominated according to the procedure of Plimmer and Phillips [1924], using 10 cc. of 0·2 *N* sodium bromate, 10 cc. of a 20 % solution of sodium bromide, and 5 cc. conc. HCl. After standing for 15 minutes, 10 cc. of a 4 % solution of sodium iodide were added and the liberated iodine was titrated with standard sodium thiosulphate. The thiosulphate solutions were carefully standardised by potassium dichromate, and frequent blank estimations were carried out with the above reagents.

Tryptophan.

cc. thiosulphate used	g. bromine absorbed
7·7	0·05760
7·9	0·05504
7·7	0·05760
7·6	0·05888

Blank experiments required 12·2 cc. thiosulphate.

1 cc. thiosulphate = 0·0128 g. bromine.

This corresponds closely to an absorption of 7 atoms of bromine by 1 molecule of tryptophan.

Tyrosine. 5 cc. of a 1 % solution were used for each determination.

cc. thiosulphate used	g. bromine absorbed
7·1	0·06784
7·2	0·06656
6·7	0·07296
7·1	0·06784

Blank experiments required 12·4 cc. thiosulphate.

1 cc. thiosulphate = 0·0128 g. bromine.

This corresponds to an absorption of 3 atoms of bromine by the molecule of tyrosine—a value, higher than that given by Plimmer and Phillips.

Histidine. 2 cc. of a 1 % solution were used for each determination.

cc. thiosulphate used	g. bromine absorbed
17·9	0·020185
17·9	0·020185
*17·3	0·025029

* This experiment was allowed to stand for 30 minutes.

Blank experiments required 20·4 cc. thiosulphate.

1 cc. thiosulphate = 0·008074 g. bromine.

This corresponds to an absorption of 2 atoms of bromine by 1 molecule of histidine.

Cystine. 1 cc. of a 1 % solution was used for each determination.

cc. thiosulphate used	g. bromine absorbed
9·85	0·03136
9·80	0·03072
9·85	0·03136

Blank experiments required 12·2 cc. thiosulphate.

1 cc. thiosulphate = 0·0128 g. bromine.

This corresponds to an absorption of 10 atoms of bromine by the molecule of cystine.

Hydrolysis and precipitation of the proteins.

Hydrolysis has been carried out for 20–24 hours with 20 % NaOH in such quantity that 4 g. NaOH were present for each g. of protein. Two cc. of butyl alcohol were added. At the end of the period the condenser was removed and the butyl alcohol driven off. Sufficient 14*N* H_2SO_4 to neutralise the NaOH was added in accordance with the directions of Folin and Ciocalteu [1927], and after shaking, the flask was allowed to cool. The solution was then made approximately *N* with respect to H_2SO_4. Filtering was carried out using a fairly large funnel (11 cm. diameter). The precipitate and paper were thoroughly washed in a beaker with 7*N* H_2SO_4. The washings were added to the solution and the final volume was made up to 100 cc. It was found that a Jena flask gave considerably less residue than a Pyrex flask, and accordingly less nitrogen was lost by adsorption.

Before precipitating, aliquot portions of this solution were titrated, and the bulk was made *N* with respect to H_2SO_4. Quantities of 8 cc. were placed in centrifuge tubes, precipitated with 15 % $HgSO_4$, washed, and decomposed in accordance with the procedure of Folin and Ciocalteu. The precipitate and the filtrate portions were made up to suitable volume and the determinations carried out on aliquot portions. Each experiment was carried out at least in duplicate.

Experiment with pure amino-acids.

A mixture containing 15·198 mg. histidine, 120·2 mg. tyrosine, 113·4 mg. tryptophan, and 167·0 mg. cystine was hydrolysed with NaOH in accordance with the above procedure. The precipitate portion was examined for total nitrogen, amino-nitrogen, using Van Slyke's apparatus, and for bromine absorption.

	mg.
Total nitrogen found	= 19·631
Calculated for 113·4 mg. tryptophan	= 15·588
Difference	+ 4·043
Amino-nitrogen found	= 9·139
Calculated for 113·4 mg. tryptophan	= 7·794
Difference	+ 1·345

Since the extra amino-nitrogen is approximately one-third of the extra total nitrogen, and is therefore in the same proportion as in histidine, it appears probable that histidine has been precipitated with the tryptophan under the conditions of experiment. It may be recalled that precipitation with $HgSO_4$ in 5 % H_2SO_4 is a regular procedure for the isolation of histidine [see also,

Onslow, 1921]. If histidine and tryptophan alone are present, the amount of histidine-nitrogen may be calculated from the formula

$$\text{Histidine-N} = 3 \times \text{total N} - 6 \times \text{amino-N}.$$

Applying this to the above figures

	mg.
Histidine-N	= 4·059
Calculated for 15·198 mg. histidine	= 4·088
	− 0·029

which would represent a loss of 0·7 %.

	mg.
The amount of bromine absorbed by this solution	= 254·08
Calculated for histidine + tryptophan	= 282·12
	− 28·04

This represents a loss of about 9·9 % on the combined values for histidine and tryptophan, and 11·6 % if the loss is merely attributed to the tryptophan. The experiment has shown that in the presence of histidine, the tryptophan may not be estimated accurately, but on account of the nitrogen figures it does not appear that either tyrosine or cystine has contributed to the error.

When the filtrate from the mercuric sulphate precipitate was examined, loss was found to have occurred.

	mg.
The amount of bromine absorbed	= 353·33
Calculated for tyrosine + cystine	= 714·72
	− 361·39
Total nitrogen found	= 29·731
Calculated	= 31·355
	− 1·624

only 49·4 % of the calculated bromine absorption and 94·8 % of the total nitrogen have been secured. If we assume that the loss is entirely due to cystine, and calculate accordingly, we find that the bromination represents a loss of 65 % cystine, although 93 % of the cystine-nitrogen is still present. Consequently, therefore, there is the probability that some cystine has escaped destruction, and may, as pointed out by Hanke [1928], interfere with the estimation of tyrosine. This interference would be experienced with bromination methods just as much as with colorimetric methods as cystine absorbs rather a large quantity of bromine (10 atoms). It will be shown that the extent of the destruction of cystine by alkali is variable.

On acidifying the alkaline hydrolysate, a strong smell of H_2S may be obtained, if the amount of cystine originally present is large. Cystine has therefore been hydrolysed by the above procedure, and directly after acidifying the hydrolysate, attempts have been made to estimate the H_2S by sucking it

through a solution of cadmium chloride and weighing the CdS formed. In one experiment where 0·2132 g. cystine was used, an amount of CdS equivalent to 0·0629 g. cystine was weighed, which represents 29·5 %. In another case using 0·2524 g. cystine, an equivalent of 0·02749 g. was recovered—a loss of 10·8 %. On another occasion the hydrolysis was continued for 100 hours and no CdS was obtained.

Continuing the usual procedure of precipitating with $HgSO_4$, bromination could be carried out with the filtrate, but no bromination was found to take place with the precipitate portion. In the above three experiments with cystine 2·8 %, 10·5 % and 5·2 % cystine have been accounted for by bromination. The results with cystine are thus very variable.

Experiments with proteins.

In view of the above results with pure amino-acids, it was of interest to carry out the procedure with a protein from which tryptophan was absent. Gelatin was selected and 1·0492 g. was hydrolysed as above, and the procedure of precipitating with $HgSO_4$ carried out.

Total N of the precipitated portion = 1·3737 mg., which if calculated as histidine = 0·5 %. Amino-N = 0·4691 mg., which equals 0·49 % histidine in gelatin, and the bromine absorption of 1 cc. = 0·0612 mg., which represents 0·46 % histidine present. Rosedale (unpublished data) has carried out a series of determinations by Van Slyke's method on this sample of gelatin and finds the value for histidine to be 0·55 %.

In another experiment tryptophan (0·0956 g.) was added to gelatin (0·8632 g.). The procedure was identical with the experiment above, and the results from the precipitate portion were:

	mg.
Total N =	14·237
Amino-N =	6·934

From the previous experiment with gelatin alone, 4·92 mg. histidine were calculated, = 1·114 mg. histidine-N. Applying the formula used in the first experiment, we get 1·107 mg. histidine-N giving a loss of 0·007 mg., which represents 0·7 %.

Bromination showed an absorption of 2·823 mg. bromine. Allowing for 5·072 mg. the amount calculated for 4·92 mg. histidine, this represents 78·588 mg. tryptophan present, which is 82·2 % of the amount originally added, and is in agreement with the experiments of Carpenter [1931].

A quantity of 0·9886 g. caseinogen was treated similarly, and the following results were obtained from the precipitate portion:

	mg.
Total N	= 9·7342
Amino-N	= 3·4626
Bromine absorbed	= 56·600

If the histidine is calculated by the formula from the nitrogen figures, we find 31·3 mg., which represents 3·1 % histidine in caseinogen. In the remainder, 1·3072 mg. tryptophan-nitrogen are present, representing 0·9 % of tryptophan in caseinogen, a figure which is low even when compared with results obtained by isolation. According to the bromination, if we allow for the calculated amount of histidine, we find only 0·86 % tryptophan. If on the other hand the presence of histidine were ignored, the bromination would give 2 % tryptophan.

It seems doubtful, however, whether tryptophan may be estimated from the nitrogen figures without taking the histidine into account, and even then it appears that low results are likely to be obtained.

Folin and Ciocalteu did not attempt to estimate tryptophan by bromine absorption and the question arises as to whether the interference of histidine might not be avoided by the use of the colorimetric determination. No series of such determinations has been made in the present work, partly because it seems evident that some of the tryptophan has been destroyed in the process of hydrolysis, and partly on account of the fact that it was found that histidine dihydrochloride imparted to the phenol reagent a greenish tint which it was considered would cause too high a reading to be made for tryptophan. The addition of sodium cyanide did not prevent this, so that any further attempt to use this method was abandoned.

Conclusions.

If tryptophan is to be estimated after alkaline hydrolysis and precipitation with $HgSO_4$, histidine, which is precipitated at the same time, must be taken into consideration. It appears that this may best be done by carrying out determinations of total nitrogen and amino-nitrogen at the same time that the colorimetric or bromination estimations are made. If the histidine is thus allowed for, it appears that the amount of tryptophan present in the solution at the time may be determined. From the results with caseinogen, it seems evident that tryptophan may be lost owing to decomposition during hydrolysis. Onslow [1921] has used baryta as hydrolytic agent, but the difficulty in applying this to such small quantities of material is that, on acidifying before precipitation with $HgSO_4$, a relatively large precipitate of $BaSO_4$ would be formed and would be difficult to wash. Onslow points out that after hydrolysis with baryta peptides are found in the mercury precipitate. This seems to have been avoided in this instance owing to the use of strong alkali. Accurate estimations of tryptophan in a protein appear to require application of Onslow's method upon larger quantities of material.

Summary.

1. An attempt has been made to estimate tryptophan and tyrosine by bromination after alkaline hydrolysis and separation by $HgSO_4$.

2. Any cystine which may escape decomposition by hydrolysis interferes with the estimation of tyrosine.

3. Histidine appears to be precipitated with tryptophan and must be taken into account. A method of doing this by means of nitrogen determinations is suggested.

In conclusion, I desire to thank Prof. Rosedale, for his interest in the work, and for much help during its progress.

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CLXXVI. EFFECT OF BACTERIOPHAGE ON THE OXIDATION-REDUCTION POTENTIALS OF *B. COLI COMMUNIS* CULTURES.

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(*Received August 15th, 1931.*)

Addition of bacteriophage to cultures of *B. dysenteriae* (Shiga) has the effect of inhibiting the fall in potential associated with the growth and metabolic activities of the organisms [Hewitt, 1931, 1]. The oxidation-reduction potentials developed in cultures of *B. coli communis* and the effect thereon of bacteriophage are described in this communication.

Methods.

The general methods and apparatus used have been described previously [Hewitt, 1931, 2]. In each experiment 8 cc. of culture medium were inoculated with 0·1 cc. of a 24-hour broth culture of *B. coli*. Dr C. H. Andrewes kindly provided a sample of bacteriophage and a culture of *B. coli communis* which was sensitive to the action of the bacteriophage. Before use the bacteriophage was subjected to several passages by adding it to 6-hour broth cultures of *B. coli* which were incubated for a further 18 hours and then filtered through small Seitz filters.

Results.

Cultures without bacteriophage.

Plain broth cultures. In peptone-infusion broth the potential of stationary aerobic cultures of *B. coli* fell rapidly, reaching its lowest level, E_h − 0·28 v., in 3½ hours. The low level of potential reached is probably due to liberation of hydrogen, visible liberation of gas in the cultures being observed after about 4 hours' incubation.

In vigorously aerated broth cultures there was a slightly longer lag period before the potential began to fall rapidly, but the lowest level of potential was reached in 3½ hours as with the stationary aerobic culture. The lowest level of potential noted in the cultures examined (− 0·36 v.) was somewhat lower than in stationary aerobic cultures. In cultures at a low potential, in which hydrogen liberation would be expected, the potential was observed to vary during the pulse of the aeration apparatus. The aeration apparatus was working at the rate of 10 cycles per minute and the potential rose during each

aeration stroke and then gradually fell again until the next stroke. The fluctuation varied from a few millivolts to nearly 0·1 v. Presumably hydrogen was being liberated rapidly and during each aeration stroke a portion of the hydrogen in the culture was displaced by air. The fact that as low a potential as − 0·36 v. could be established in a vigorously aerated culture illustrates the intense metabolic and enzymic activity displayed by *B. coli communis*.

Since *B. coli* contains catalase it was not surprising to find that no evidence could be obtained of the establishment of the high potentials corresponding to peroxide formation.

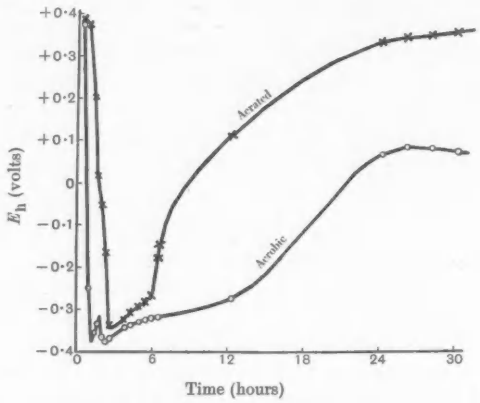

Fig. 1. Glucose broth cultures of *B. coli*.

Glucose broth cultures (Fig. 1). The fall in potential in 1 % glucose broth cultures was the most rapid yet recorded. In stationary aerobic cultures the potential had reached its lowest level (− 0·38 v.) in 2 hours. The most rapid fall in potential observed was 0·65 v. in 30 minutes. The kink in the potential-time curve which occurred in this case after 1 hour's incubation has been observed also with haemolytic streptococci.

In aerated glucose broth cultures the fall in potential was only slightly less rapid than in stationary aerobic glucose broth cultures, but after the logarithmic phase of growth the potential rose very rapidly.

Effect of bacteriophage on potentials.

0·1 cc. or 0·25 cc. of bacteriophage was added to each cell containing 8 cc. of culture.

Plain broth cultures. In stationary aerobic broth cultures containing 0·1 cc. of bacteriophage (Fig. 2) the potential fell to the level + 0·05 v. where it was maintained for some hours. The potential then began to fall again and

after 24 hours' incubation the low level reached in cultures without bacteriophage had been attained.

In aerated broth cultures containing 0·1 cc. of bacteriophage (Fig. 3) there was a delay of 6 or 7 hours before the potential fell below the level + 0·2 v. but after 12 hours' incubation the potential had reached almost as low a level as in aerated cultures not containing bacteriophage.

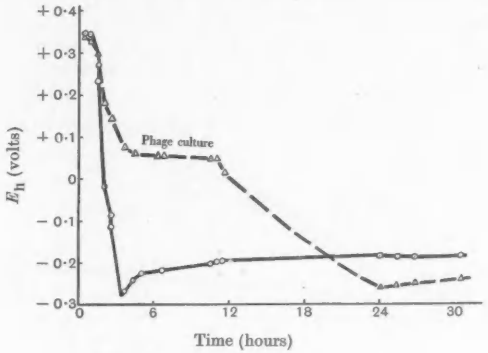

Fig. 2. Aerobic broth cultures.

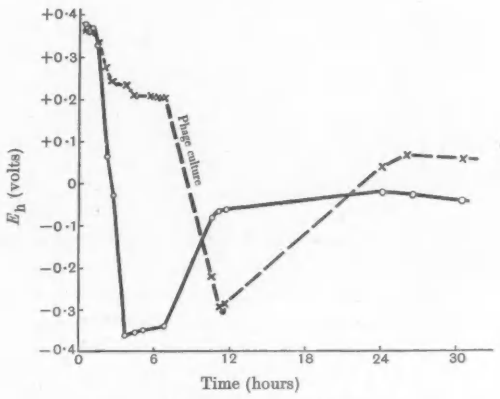

Fig. 3. Aerated broth cultures.

Glucose broth cultures. In stationary aerobic 1 % glucose broth cultures containing 0·25 cc. of bacteriophage (Fig. 4) the initial fall in potential was as rapid as in cultures without bacteriophage, but after this initial fall to a low level there was a rapid rise of potential. Later there was a further fall in potential followed by another rise and fall. Both growth and gas formation are visible in 6-hour glucose broth cultures to which bacteriophage has not

been added, but in cultures containing bacteriophage neither growth nor gas formation is observed until much later (see p. 1646).

In aerated glucose broth cultures containing bacteriophage (Fig. 5) the initial fall in potential was comparatively slight and not as rapid as in cultures without bacteriophage. The initial fall in potential was followed by a rise

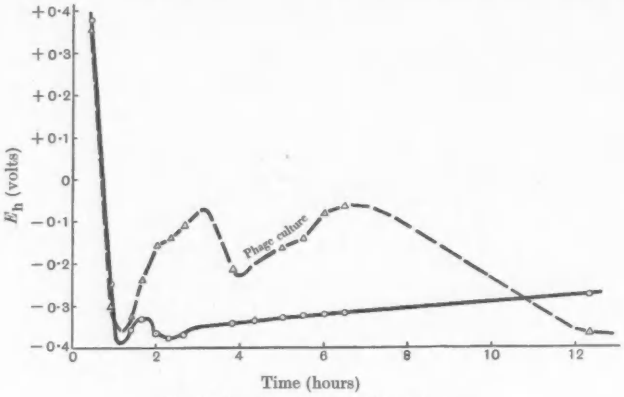

Fig. 4. Aerobic glucose broth cultures.

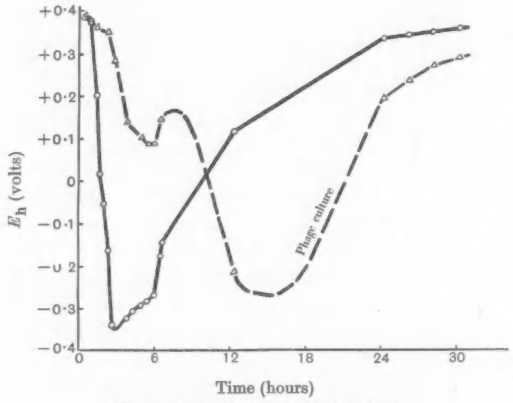

Fig. 5. Aerated glucose broth cultures.

and a subsequent fall to a low level when growth in the culture became visible. The reducing activity of the non-proliferating organisms was not sufficient to overcome the oxidising effect of the extra oxygen supplied to the culture by means of the aeration apparatus, but subsequently, when the organisms had proliferated sufficiently to produce visible growth in the culture, the usual fall in potential occurred.

Discussion.

The electrode potential in cultures of *B. coli* attains lower levels than have been described with other bacteria in this series of communications. These low potentials are associated with the mechanism possessed by *B. coli* for the liberation of hydrogen in its cultures. It may be noted that the potential produced at an electrode in the presence of one atmosphere pressure of hydrogen at p_H 7·0 is − 0·43 v. (at 37°) and in the present series of observations a potential of − 0·36 v. was observed in a culture subjected to aeration. In addition to the attainment of low levels of potential the rate of fall of potential is very rapid in *B. coli* cultures. In 1 % glucose broth cultures the fall is particularly rapid and the minimum level of potential (*ca.* − 0·37 v.) is reached in just over an hour.

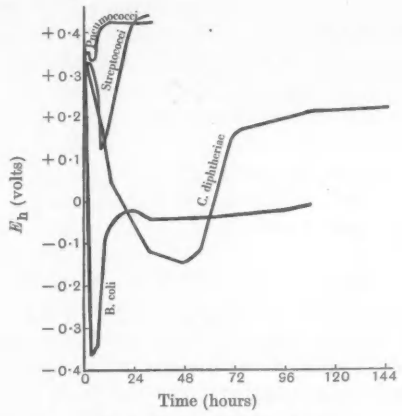

Fig. 6. Aerated broth cultures.

The characteristic behaviour of *B. coli* can be seen in Fig. 6 which gives the electrode potential time curves of aerated broth cultures of three classes of bacteria. Peroxide-forming organisms are represented by a pneumococcus and haemolytic streptococcus, catalase-containing organisms by *C. diphtheriae* and gas-forming organisms by *B. coli*.

The appropriate bacteriophage almost completely inhibits the fall in potential of *B. dysenteriae* (Shiga) cultures [Hewitt, 1931, 1], but in the case of *B. coli* the fall in potential is merely delayed and the potential eventually reaches approximately the same low level in the presence or absence of bacteriophage. The same observation was made with regard to the effect on the growth of the cultures. In the presence of the samples of bacteriophage used in these experiments no growth of *B. dysenteriae* (Shiga) was observed, but with *B. coli* visible growth was merely delayed when bacteriophage had been added. The results reported support the view previously expressed in

the case of *B. dysenteriae* [Hewitt, 1931, 1] that bacteriophage appears to have no effect on the oxidation-reduction potential of cultures except that it inhibits the usual fall in potential accompanying the proliferation and metabolic activities of the bacteria.

An interesting phenomenon was observed in stationary aerobic glucose broth cultures to which bacteriophage had been added. Despite the presence of bacteriophage the potential fell initially as rapidly as in its absence although the growth of the organisms was delayed. After this initial fall the potential rose fairly rapidly but fell again when active proliferation of the bacteria occurred. It is evident that the enzymes present in the bacteria inoculated into the medium were able to activate the glucose present and to establish temporarily intense reducing conditions. The re-establishment of low levels of potential occurred only when the numbers of bacteria present had increased greatly. In aerated glucose broth cultures containing bacteriophage the activity of the bacterial enzymes was insufficient to produce a large initial fall in potential and intense reducing conditions were not established until visible growth had occurred in the culture.

The general effects of the appropriate bacteriophages on the potentials of cultures of *B. dysenteriae* (Shiga) and *B. coli* are similar except that in the case of the Shiga bacillus the bacteriophage completely inhibits growth and the fall in potential, whilst in the case of *B. coli* proliferation and the fall in potential are merely delayed.

Summary.

1. The potential of *B. coli* cultures falls to a lower level and much more rapidly, especially in the presence of glucose, than in the case of other bacteria investigated. This is attributed to liberation of hydrogen in the culture and to a generally intense enzyme activity.

2. In the presence of bacteriophage the fall in potential and also the proliferation of the bacteria are delayed but not completely inhibited as in the case of *B. dysenteriae* (Shiga).

3. The effect of bacteriophage on the oxidation-reduction potential follows the effect on the proliferation of the bacteria, except that in aerobic glucose broth cultures of *B. coli* containing bacteriophage there is an initial fall in potential without appreciable bacterial growth, possibly due to activation of glucose by the enzymes of non-proliferating bacteria.

The author is indebted to Dr C. H. Andrewes for the culture and bacteriophage used and to Dr R. G. White and Dr E. W. Todd for their help and interest.

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CLXXVII. THE INFLUENCE OF SUNLIGHT ON MILK.

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Lojander [1925] noticed that exposure to sunlight decreased the time required to decolorise methylene blue in milk containing bacteria. The present author [1930] showed that sunlight caused a decoloration of methylene blue in milk independently of bacteria, and produced evidence in support of the theory that the reaction was accompanied by an oxidation of some portion of the milk fat.

Hammer and Cordes [1920] and Frazier [1928] had previously observed that whole milk exposed to sunlight developed a "cardboard" or tallowy flavour due to the oxidation of the milk fat. From the evidence available it seems probable that the two phenomena are based on the same chemical reaction so far as the fat is concerned, and that the presence of methylene blue in milk exposed to sunlight merely results in a visual indication of the intensity with which the fat oxidation is taking place. The author has observed that milk samples, exposed to sunlight with or without methylene blue, develop the tallowy flavour described by the American workers, and since neither the tallowy flavour nor the reduction of methylene blue occurs in milk from which the fat has been removed, it is almost certain that the two happenings are dependent upon one reaction—an oxidation-reduction phenomenon. The reaction appeared to be of sufficient interest to warrant a more detailed examination.

It has been shown by Clark *et al.* [1928] that the potential induced on an electrode of a noble metal immersed in a solution indicates the reducing or oxidising tendency more precisely and over a greater range than do the changes in colour undergone by a small amount of dye (such as methylene blue). The potentiometric method was applied, therefore, in an attempt to follow more fully the changes which take place in milk exposed to sunlight. Gold electrodes were immersed in tubes of milk. KCl-agar bridges connected the milk sample with a quinhydrone half cell prepared according to Veibel [1923]. The E.M.F. values given by such complete cells were measured at intervals by means of a potentiometer, and the E_h values calculated from the readings were plotted against time intervals. The milk used for the deter-

mination of the effect of sunlight was fresh and contained so few bacteria that it gave no appreciable change in E_h value within 7 hours when kept in darkness at 37°. In order to prevent the fat from rising too rapidly in the milk, the electrodes were moved gently up and down with a pumping action before taking each reading on the potentiometer, but they were never withdrawn wholly from the milk during the course of the experiment.

Whole milk.

When whole milk was exposed to sunlight, considerable changes in E_h were soon observed coincident with the decoloration of methylene blue in

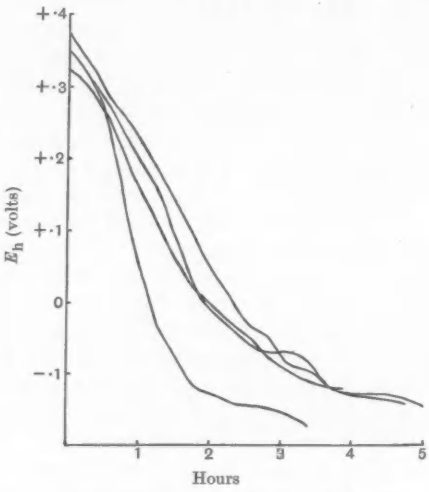

Fig. 1. Whole milk in bright sunlight.

duplicate samples also exposed to light. Fig. 1 shows the curves obtained in several typical experiments.

The fact that a fall in E_h occurs in the absence of methylene blue indicates that the dye is not essential to the reaction and that there must be some reversible system in milk capable of acting as a hydrogen acceptor. Clark and Cohen [1928] have shown that this is also the case in the Schardinger reaction. The time required to bring about the change and also the extent of the change varied considerably with different milks, with the intensity of the sunlight, and also with the particular electrode used. It soon became evident that the reducing potentials induced in the milk were very poorly poised and that any attempt to make accurate measurements would be useless. Different electrodes in duplicate samples of milk under apparently indentical conditions often showed differences of 50 millivolts in the potentiometer readings. The low

capacity of the reducing system formed during the fat oxidation was also evidenced by the fact that the presence of methylene blue (in dilution of 1 in 100,000) in milk in which the electrode was immersed resulted in a very definite tendency for the fall in potential to be checked in the region where methylene blue reduction occurs. In spite of this unavoidable inaccuracy, however, it was possible to gain some information from the general form of the curves which in a large number of experiments was fairly constant.

The form of curve obtained with whole milk exposed to sunlight is quite different from that obtained by plotting the changes of oxidation-reduction potential with time in milk in which bacteria are present in large numbers. In such cases the potential shows only a slight fall for some time and then drops very suddenly to a negative E_h value. The difference is easy to understand, for, when the action is due to bacteria, there is a rapid increase in the amount of enzyme (due to the multiplication of the organisms) during the course of the experiment, whereas in the action induced by sunlight there can be no increase in the amount of catalyst unless the intensity of the light increases markedly or the reaction is autocatalysed. The shape of the curve in the present experiments indicates that autocatalysis does not occur. In both types of reaction, however, the lower limit of E_h value reached appears to be about $-0{\cdot}2$ v.

An apparent anomaly in the course of the reaction which was often evident was that in bright sunlight the methylene blue underwent decoloration while the E_h of the milk was still over $+0{\cdot}2$ v., whereas the normal range for decoloration in aqueous solution is $+0{\cdot}07$ to $-0{\cdot}01$ v. at the p_H of milk. The reason for this has not been elucidated, but it seems very probable that, while the E_h of whole milk is changing rapidly, the potential on the electrode may lag in its indication of the conditions in the medium. This is especially likely to occur in the present case where the reaction responsible for the development of the reducing potential is taking place at the interface between two phases—the fat and the plasma. Some evidence that this lag in adjustment takes place was obtained by removing a tube of milk from bright sunlight to darkness midway in the reaction. When this was done, the potential on an electrode immersed in the milk continued to fall for some time, although it ceased to fall long before the potential in a duplicate tube exposed to sunlight the whole time. It seems more likely that in darkness the continued fall was due to an adjustment of the electrode to the actual conditions in the medium than that the reducing tendency continued to increase after the stimulus of sunlight was removed. Where the fall in potential was more gradual or less fat was present (as will be mentioned later), this decoloration of methylene blue at a potential apparently too high did not occur. In these cases also, removal of the tube from the influence of sunlight resulted in an immediate check in the fall in potential. It is suggested, therefore, that where the E_h falls more gradually or the medium is more nearly homogeneous, the potential on the electrode gives a more accurate indication of the conditions

in the medium from moment to moment. If this be the true explanation, it becomes evident that, under some conditions, a dye may give a truer indication of the oxidation-reduction potential of a solution than the potential on an electrode.

In early experiments the milk was maintained at 37° to 40° during exposure to sunlight. Owing to the inaccuracy of the potentiometric method, no attempt was made to determine the temperature coefficient of the reaction. It was found, however, that exposure of milk at room temperature resulted in a slowing of the reaction barely outside the limits of experimental error due to differences between electrodes. All later experiments were therefore performed at room temperature.

The reaction obtained with heated milk confirmed the results reported previously by the present author in that there was no essential difference from that obtained with raw milk.

Separated milk.

The results obtained by the exposure of separated milk to sunlight and measurement of the reducing potential were quite different from those obtained with whole milk and were not so regular. Many samples showed little change in E_h during the period of exposure to sunlight, this corresponding to the absence of change in methylene blue under such conditions. Other samples gave a decided fall similar to that obtained with whole milk, but the potential never fell much below zero E_h and then always showed a tendency to rise again to an E_h of between $+0{\cdot}2$ and $+0{\cdot}3$ v. This might be explained by assuming that the slight amount of residual fat (about 0·40 %) tended to undergo oxidation under the influence of the sunlight. The subsequent drift to a more positive potential can only be explained by assuming the presence in milk of some slowly reacting reducible system of large capacity, which in the oxidised form has an E_h of between $+0{\cdot}2$ and $+0{\cdot}3$ v. The presence of such a system in milk seems probable by reason of the fact that most milk samples, in the absence of influences such as sunlight and bacteria, attain equilibrium with a gold electrode at about this value of E_h. Fig. 2 gives several typical curves obtained with separated milk.

Although the curves obtained with some samples fell through the range where methylene blue reduction occurs, the dye was never reduced completely in separated milk. The reducing system had such a low capacity that the dye, when present, prevented by its poising action the extreme fall in E_h indicated in some of the curves. As has been mentioned before, a paling in colour sometimes took place with a subsequent deepening in colour as the potential rose again. The separated milk, moreover, never showed a continued fall in potential when the stimulus of sunlight was removed, and it is probable that the electrode was able to indicate the true potential of the medium more accurately from moment to moment. The occurrence or non-occurrence of a fall in potential in different batches of separated milk could not be correlated

with slight differences in fat content, and it is impossible at present to account satisfactorily for this variation in behaviour.

The effect of ultra-violet radiation.

Although in the foregoing experiments the sunlight passed through at least two layers of glass, the use of an ultra-violet spectroscope showed that some ultra-violet rays in sunlight were capable of reaching the milk.

In order to determine whether ultra-violet radiation was responsible for the stimulation of fat oxidation, experiments such as those already described were performed, replacing sunlight by radiation from a mercury vapour lamp. The tubes of milk were exposed at distances from the lamp varying from 1 ft.

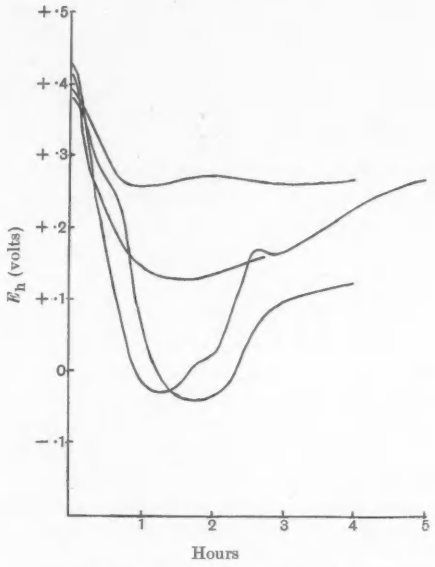

Fig. 2. Separated milk in bright sunlight.

to 6 ft. Even at the shortest distance, no change in blue colour was observed in milk containing the dye, and only a very slow change in potential was observed until about 6 hours had elapsed from the beginning of irradiation. It appeared to take at least three times as long to produce any effect by means of ultra-violet radiation as by sunlight. Another difference was that the blue did not begin to fade until the E_h had fallen below zero, and it seems most probable that, owing to the slowness of the changes taking place, the electrode was able to maintain its adjustment with conditions in the milk, thus indicating more exactly the true potential from moment to moment than was the case in milk exposed to sunlight and undergoing rapid changes. Separated

milk gave results similar to those obtained in sunlight, but again the course of any reaction which occurred was much more prolonged. The agent inducing oxidation of fat in milk is therefore not ultra-violet radiation (or at least any of the ultra-violet rays most characteristic of the radiation from a mercury vapour lamp), but more probably some portion of visible light of which there is less in the radiation of the mercury vapour lamp than in sunlight. Trials with radiation from electric lamps were negative so far as any effect on oxidation of fat or reduction of methylene blue were concerned, and at present it is therefore impossible to define more accurately the particular type of radiation responsible for the reaction.

Summary.

The oxidation of fat in whole milk under the influence of sunlight causes the development of a reducing potential, which can be detected either by its effect on methylene blue or by measurement electrometrically. The potential developed is poorly poised and of small capacity, hence experimental error in the measurement of potentials is great. Separated milk in many cases undergoes no change under the influence of sunlight, but some samples show a fall in potential which is not at present satisfactorily explained. When such a fall in potential occurs in separated milk there is a subsequent drift to more positive potentials. This suggests the presence in milk of a slowly acting reversible system which tends to maintain the E_h at a value between $+0{\cdot}2$ and $+0{\cdot}3$ v. It has not been found possible to reproduce the effect of sunlight by the use of either ultra-violet radiation or of radiation from electric lamps.

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CLXXVIII. A METHOD FOR THE DIRECT DETERMINATION OF UREA IN URINE.

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THE method introduced by Marshall [1913] is not used extensively, probably because of the length of time necessary for the completion of the action of the urease.

By using a stronger enzyme preparation and by maintaining the fluid under optimal conditions of reaction, it is possible to complete the estimation in about 15 minutes.

The principle of the method in brief is as follows. The urine is treated with warm water and some phenolphthalein, the urease preparation is added, and the mixture is titrated with 0·1 *N* HCl, at such a rate as just to discharge the red colour. This prevents the loss of ammonia and maintains a reaction of about p_H 8·2, which is about the optimum for urease acting on dilute solutions of urea. After about 10 minutes the mixture is treated with mercuric chloride and some methyl red. The addition of the HCl is continued until a red colour is obtained which matches that given by a mixture of the same amount of urine, mercuric chloride, enzyme preparation and methyl red which is titrated from a separate burette to give a red colour. In this case the mercuric chloride is added before the urease, which is unable to act in the presence of the mercury. A certain amount of ammonium chloride is added to the control to ensure uniform salt conditions for a correct adjustment of the final p_H. The difference between the amounts of HCl required for the two solutions is a measure of the urea in the urine taken.

SOLUTIONS REQUIRED.

(*a*) 0·1 *N* HCl, in two burettes.
(*b*) Mercuric chloride. 0·5 % made up to 100 cc.
(*c*) Ammonium chloride, 10 %.
(*d*) Phenolphthalein. A saturated solution in 50 % alcohol.
(*e*) Methyl red. 0·02 % in 60 % alcohol.
(*f*) Urease solution. This is best made from "Arlco" Jack Bean Meal[1].

The simplest method is to place 4 g. of the meal in a mortar and rub it up

[1] This is prepared by The Arlington Chemical Co., Yonkers, N.Y., and is stocked by Baird and Tatlock, 14 Cross St., E.C. 1.

into a cream with 10 cc. of distilled water. Wash this into a beaker with more water until 100 cc. have been added. Stir well and allow to stand for some minutes. Pour off from a deposit of starch and other insoluble material. Such a preparation gives a considerable precipitate at the final end-point of the titration, but this does not materially interfere with the accuracy. The following method gives an almost clear solution. A 4 % suspension of the meal is made as above with the exception that the last 50 cc. of water are warmed to about 60° before being added. The whole is treated with 4 cc. of 0·1 *N* HCl, mixed well and filtered through a pleated paper. A water-clear filtrate is obtained. This gradually deposits a slight precipitate, which should not be filtered off.

Method of estimation. Into two similar 100 cc. beakers, labelled *A* and *B*, measure 3 cc. of the filtered urine. To each add about 20 cc. of distilled water at about 60° and 5 drops of the phenolphthalein. To *A* add 10 cc. of the enzyme preparation. As soon as this goes red add some 0·1 *N* HCl from a burette, and continue to do this at intervals, so as just to prevent a red colour developing. Care should be taken not to add too much acid, as this inhibits the action of the enzyme. As a rule it will not be necessary to add any acid after about 5 minutes. It is, however, advisable to allow the mixture to stand for about 12 or 15 minutes, depending on the concentration of the urea. During the interval place 1 cc. of the mercuric chloride and 1 cc. of the ammonium chloride in *B*. Mix well and then add 10 cc. of the enzyme preparation. Now add 20 drops of the methyl red (measured with a dropping pipette). With most samples of urine the mixture is yellow. Add 0·1 *N* HCl from a second burette until a full red colour is obtained, enough water being added to make the volume slightly greater than that in *A*. At the end of 12 or 15 minutes' digestion the fluid in *A* is treated with 1 cc. of the mercuric chloride, and 20 drops of methyl red. The contents of *A* are then titrated with the HCl until the colours of the fluids in the two beakers match, the volumes being kept constant by the addition of water to one or the other. It will be found that the red colour produced in *A* by the addition of acid tends to fade a little at first, owing to the rather slow establishment of equilibrium of the carbonic acid, but the two colours can be matched with great exactitude if a little care is given to this point. It is advisable to add enough acid to *B* to produce a rather bright red tint, otherwise the result may be a trifle low, owing to the effect of the carbonic acid in *A*. But the effect of this is negligible if the end-point of the titration is sufficiently acid.

Calculation. As 1 cc. of 0·1 *N* HCl is equivalent to 3 mg. of urea, the number of cc. of the acid added to *A* less that added to *B* gives the urea in g./litre.

Accuracy of the results. Duplicate estimations on the same sample of urine agree to 1 % with the filtered extract, and to about 2 % with the untreated suspension.

The results agree with those obtained by estimating the ammonia and the ammonia- *plus* urea-nitrogen by Van Slyke's methods [Van Slyke and Cullen, 1914] with the same degree of accuracy.

Theoretical results are obtained with solutions of urea up to 5 %, and also with urines to which urea has been added to make a total of 5 %.

With urines obtained when making the urea concentration test of renal efficiency, it is advisable to use 2 cc., and to multiply the cc. of acid used for *A* less that used for *B* by 1·5 to give the urea in g./litre.

SUMMARY.

A method is described for the estimation of urea in urine which can be completed in 20 minutes, and which is accurate to within 2 %.

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CLXXIX. THE ACCESSORY FACTOR NECESSARY FOR THE GROWTH OF *NEMATOSPORA GOSSYPII*.

I. THE CHEMICAL NATURE OF THE ACCESSORY FACTOR.

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(*Received August 20th, 1931.*)

Since the earliest attempts were made to cultivate fungi on synthetic nutrient media, cases have been met with in which certain fungi were unable to grow in such media in the absence of small amounts of extracts prepared from natural products. The best known instance of this difficulty of culture is that of ordinary yeast, *S. cerevisiae*, which has been shown to need the presence of a small quantity of "bios" to enable healthy growth to take place on salt-sugar media. The nature of "bios" remains undetermined, although the problem has engaged the attention of many workers for some 30 years.

In his studies on *Fusarium*, Brown [1925] devised a synthetic medium on which the growth of *F. fructigenum* was quite comparable with that on potato decoctions. This medium had the composition: glucose, 2 g.; asparagine, 2 g.; potassium phosphate, 1·25 g.; magnesium sulphate, 0·75 g.; agar, 15 g.; water, to 1000 cc. The same medium was found suitable for the cultivation of a large number of fungi, but cases were met with in which no growth could be obtained. Among the fungi unable to utilise the medium were certain varieties of *Nematospora*, including that investigated in the present instance, *N. gossypii*. The study of the nutritional needs of this organism was continued by Farries and Bell [1930], who were able to show that Brown's medium could be made entirely suitable if the asparagine were replaced by peptone or by the mixed hydrolysis products of certain proteins. They found further that the hydrolysis mixtures prepared from different proteins were not all equally suitable, those obtained from edestin and gelatin, for instance, being quite useless. Nor could the fungus utilise mixtures of all types of pure amino-acids, in the proportions in which they occur in a typical protein.

Farries and Bell concluded that, in general, the mixed hydrolysis products of purified proteins were valueless as a source of nitrogen for this fungus, whereas the hydrolysis mixtures from the crude proteins were entirely satisfactory, and gave good growth. They separated from egg-white, and also from

crude caseinogen, an "accessory factor," itself probably non-nitrogenous, the presence of which was essential for the utilisation of simple nitrogenous substances by *N. gossypii*. This "accessory factor" was readily obtained from egg-white by precipitation of the albumin with alcohol, the accessory being soluble in 80 % alcohol. Even the hydrolysis products of gelatin could readily be assimilated by the fungus in the presence of small amounts of this accessory.

The chemical nature of the accessory factor was not determined by Farries and Bell, although they made a number of experiments in an attempt to characterise the substance. They concluded that it was an organic compound, but not a polysaccharide, sugar, sugar alcohol or sugar acid, fat, ester or lipoid. They considered that it had acid properties, since it was precipitated by copper hydroxide and lead hydroxide. Whether the substance was nitrogenous could not be established; that it was not a basic amino-acid was shown by its non-precipitability by phosphotungstic acid or mercuric chloride. The most active concentrate prepared from egg-white by Farries and Bell had a very low nitrogen content.

It was in an attempt to gain more information as to the chemical nature of the "accessory factor" that the present work was undertaken.

The strain of fungus with which the experiments were carried out was that used by Farries and Bell, and the technique adopted was that which had been carefully standardised by these workers, and which is described briefly below. The use of egg-white as a source of the accessory factor was abandoned early in the work, for reasons given below, and, after a brief survey of other possible sources of the factor, it was decided to prepare the concentrates from lentils. Subsidiary tests were made with concentrates prepared from another plant source (lupin seedling extract) and from an animal source (ox-heart) and the results were found to be comparable. There was therefore no reason to suppose that egg-white would have proved any exception in respect of the nature of the accessory factor which it contained.

Experimental.

Method.

The organism used throughout the work was a strain of *N. gossypii* which had been isolated by Brown from diseased cotton bolls (East African). This strain gave vigorous growth, and when cultivated on solid media formed a tough mat of mycelium on the surface of the medium. The mat could be peeled off completely without disturbing the medium, and it was shown by Farries and Bell [1930] that the amount of mycelium remaining within the medium was only 3 % of the total mycelium. The "dry weight" method of estimating the growth of the fungus could therefore be adopted, the percentage error due to incomplete removal of the fungus from the medium being constant.

In order to ensure a maximum degree of regularity in the different cultures, the details of routine were standardised. The basal medium used had the following composition: glucose, 2 %; potassium phosphate, 0·5 %; magnesium

sulphate, 0·25 %; agar, 2 %. The nitrogenous constituent, from whatever source, was supplied in a concentration equivalent to 0·3 % N (see below).

The Petri dishes used were 3 in. in diameter, and the amount of medium added to each was 25 cc. A mycelium inoculation of the fungus (of which stock cultures were maintained on glucose-peptone agar) was placed at the centre of each plate, and the cultures were incubated at 30° for 6–7 days. At the end of this period the superficial mat of mycelium was peeled off the agar, dried on a tared filter-paper in a steam-oven for 48 hours and weighed. In general, the plates were treated in pairs, so that the "dry weight" figures quoted below represent the combined weight of mycelium from 2 plates.

As stated above, the nitrogenous constituent in the medium was supplied in a concentration equivalent to 0·3 % N. The main supply of nitrogen was provided by adding the appropriate amount of an amino-acid mixture prepared from gelatin by hydrolysing with 22 % sulphuric acid, removing excess of acid by baryta, and concentrating the filtrate to a known strength. Since the amounts of lentil and other concentrates tested themselves contained nitrogen, this was estimated and allowed for in adding the amino-acid mixture. In the tables given below, the term "standard basal medium" is intended to represent the salt-sugar mixture plus the 0·3 % N (from the combined sources, amino-acids and concentrate) referred to above.

The reaction of the medium was adjusted to p_H 6·8–7·0 before inoculation. The work of Farries and Bell had shown that the fungus possessed a comparatively wide range of growth, with an optimum at p_H 7·0.

In all cases where growth was found below normal, strict controls were set up to test for toxicity in the medium; this was particularly necessary, in view of the possibility of the presence of small traces of lead, barium, *etc.* in the media. In these cases, therefore, portions of the concentrate in question were added to a medium known to give good growth; if the resulting growth were at least as good as that on the standard medium, it was assumed that lack of accessory factor, and not toxicity in the concentrate, was responsible for lack of growth in the absence of the standard concentrate.

The effect upon growth of different amounts of concentrate was investigated, and it was found that addition of a small amount of concentrate produced a relatively large increment in growth, further small additions giving proportionate increases. A point was reached, however, after which the addition of a further large supply of concentrate produced only a small increment in growth, such as might often be accounted for by addition of actual food material present in the portion of concentrate added. If a curve were plotted showing the weight of concentrate added against the weight of mycelium produced, a fairly sharp change of gradient was observed where addition of more concentrate no longer produced great increase in growth. This point was taken as indicating the "critical amount" of the concentrate necessary and all comparisons of activity were made on the basis of this "critical amount" of concentrate. Examples of the method are given below.

Tests for activity in known substances.

Before attempting to fractionate the concentrates prepared from various sources, it was thought worth while to ascertain whether certain substances, which had from time to time been suggested as growth-promoting accessories by workers on animal nutrition and bacterial metabolism, could supply the essential nutrient for the growth of *N. gossypii*.

Nucleic acid (yeast), adenine sulphate, guanine hydrochloride, mannitol, inositol and inositol plus traces of Fe or Cu were tested, but in no case was any appreciable effect observed.

Sources of the "accessory factor."

As stated above, the material from which Farries and Bell prepared their active concentrate was egg-white, which they fractionated into "albumin" and "egg-extract" by precipitation with alcohol. They found that the albumin, whether hydrolysed or not, did not give any measurable growth of *N. gossypii*, but when supplemented with very small amounts of the "egg-extract" gave copious growth. According to their figures, the growth on albumin alone was 1·5 % of that on albumin plus "egg-extract." The first attempts to prepare concentrates of the active factor followed the methods given by Farries and Bell. It was found that the original egg-white (hydrolysed) gave good growth, and a sample of crystalline albumin prepared therefrom only negligible growth, thus confirming the results of Farries and Bell. On the other hand, the preparation of the "egg-extract" in good yield was found to be a matter of some difficulty, the active factor being retained somewhat tenaciously by the albumin when the latter was precipitated. On account of this difficulty in manipulation, and the expense involved in using egg-white as a starting material, other sources of the accessory factor were sought. At the same time, a number of substances were tested for the presence of the factor, so that a brief survey of the distribution of the factor among a variety of products might be obtained.

(*a*) *Cotton plant.* As the natural habitat of the fungus is in the bolls of the cotton plant, it was thought worth while to see whether other parts of the plant would also yield the accessory factor. Cotton leaves (about 50 g.) were ground to a pulp, and treated with 3 volumes of 95 % alcohol; the filtrate was evaporated, taken up in water and made up to 50 cc. Sufficient of this solution to correspond with 0·5 g. of solid matter was incorporated in 50 cc. of the medium and growth tested. The results of this and other tests on various extracts are summarised in Table I.

(*b*) *Potato.* 300 g. potato were steamed for 3 hours and the aqueous extract was precipitated with alcohol (3 volumes). The portion soluble in 75 % alcohol was taken up in water, and varying amounts were plated with the basal medium according to the usual procedure.

(*c*) *Lentils.* 300 g. portions of lentils were treated in the same way as the potatoes.

(*d*) *Lupin seedlings.* A portion of an extract of etiolated lupin seedlings which was available was treated with alcohol, as in the case of other extracts, to remove most of the inorganic salts, *etc.* The filtrate, after removal of alcohol, was tested for activity.

(*e*) *Yeast extract.* A sample of zymin was prepared from brewer's yeast by the acetone method. This was boiled with water for 30 minutes, and the solid material removed on the centrifuge. The extract was concentrated, and portions were incorporated in the basal medium. Similar tests were also made with a commercial yeast preparation (marmite), this being used without further treatment.

(*f*) *Ox-heart.* Preparations of ox-heart were used by Reader [1928] as a source of the factor promoting growth of *Streptothrix corallinus*; accordingly, a tryptic digest of fresh ox-heart was prepared according to the method described by Reader, except that a much longer period of digestion was found necessary to produce a mixture not giving the biuret reaction. The soluble portion of the resultant mixture was made up to a known concentration, and portions were added to the basal media.

(*g*) *Fibrin.* A sample of commercial fibrin (B.D.H.) was digested in acid solution with pepsin for 1 month. The insoluble residue was filtered off, and the filtrate tested for activity.

Table I.

Extract tested	Weight of concentrate in 50 cc. medium g.	Dry weight of mycelium (2 plates) g.
Cotton leaves	0·5	0·15
Potato	0·1	—
	0·2	0·05
	0·3	0·11
	0·4	0·14
	0·5	0·18
Lentils	0·1	0·28
	0·2	0·31
	0·3	0·33
	0·4	0·35
	0·5	0·375
Lupin	0·05*	0·06
	0·1	0·12
	0·2	0·195
	0·3	0·25
	0·4	0·295
	0·5	0·32
Yeast	0·05	0·08
	0·075	0·10
	0·1	0·13
(Marmite)	0·075	0·11
	0·15	0·185
Ox-heart digest	0·1*	0·08
	0·3	0·19
	0·5	0·25
Fibrin digest	0·1	—
	0·5	—

* Ash-free organic matter.

The relative activities of the concentrates prepared from different materials were clearly indicated by these tests. The greatest activity was shown by lentil extracts, while lupin extract, yeast and ox-heart were fairly rich in the factor. Potato was relatively poor, and fibrin deficient in the factor. (This deficiency was noted by Farries and Bell.) To the list of substances rich in the accessory must be added those recorded by Farries and Bell, *viz.* egg-white, impure caseinogen and commercial peptone.

In view of the results thus obtained, it was decided to use lentils as the starting material in attempts to prepare more active concentrates than those hitherto dealt with.

Preparation of the lentil concentrate.

About 50 kg. of lentils, dealt with in portions of 500 g., were steamed with twice their weight of water for 3 hours, the residual solid was removed, and the extract evaporated to about 300 cc. The addition of 3 volumes of alcohol gave a large precipitate, containing inorganic salts, proteins, *etc.* This was filtered off, and the filtrate again evaporated to a small bulk, and precipitated with alcohol. The filtrate at this stage was evaporated to a syrup and treated with dry acetone, thus granulating the substance. The solid was dried *in vacuo*, when it was obtained as a light brown, somewhat hygroscopic powder, containing 2·88 % N. The yield from 50 kg. of lentils amounted to about 100 g. Its activity was similar to that of the sample obtained on the small scale, as described above—0·1 g. in 50 cc. medium gave 0·28 g. dry mycelium.

When different amounts of the lentil preparation were incorporated in the medium, the corresponding growths obtained were those shown in Table II.

Table II.

Weight of lentil concentrate per 50 cc. medium	Dry weight of mycelium
g.	g.
0·01	—
0·025	0·06
0·05	0·13
0·075	0·195
0·10	0·28
0·20	0·31
0·30	0·33
0·40	0·35
0·50	0·375

It was evident from these results that the "critical dose" of the lentil concentrate lay in the region of 0·1 g. per 50 cc. of medium. Accordingly, all tests were made on a basis of 0·1 g. original concentrate per 50 cc. medium. Thus it was necessary at each stage of the fractionation to determine the weight of substance in each fraction, corresponding to 0·1 g. of the original substance; since there was a small loss of total substance at every stage of the fractionation process, it was assumed that the fractions (*e.g.* filtrate and ppt.) suffered the same proportionate loss.

Fractionation of the lentil concentrate.

In the course of their attempts to fractionate the "egg-extract," Farries and Bell found that the active factor was precipitated by copper hydroxide or lead hydroxide; accordingly, the first attempts to fractionate the lentil concentrate were made on these lines. It was found, however, that although the active substance was apparently precipitated by these reagents, it was impossible to recover it from the precipitates, probably on account of the fact that nearly all the active substance was adsorbed on the copper (or lead) sulphide produced when the precipitates were decomposed. For this reason these experiments were not proceeded with. A number of preliminary tests indicated that a considerable degree of concentration could be obtained by treatment of the lentil extract in the following manner.

(i) Removal of basic substances (inactive) by phosphotungstic acid.

(ii) Removal of the majority of organic acids as barium salts, by precipitation with baryta and alcohol.

(iii) Precipitation of the active factor as a mercury compound by Neuberg and Kerb's reagent (mercuric acetate and sodium carbonate).

Accordingly, 80 g. of the lentil preparation were submitted to the above process. The precipitation with phosphotungstic acid was carried out in the ordinary way, in the presence of 5 % sulphuric acid, which was subsequently removed from the filtrate by baryta. The activity was found to be confined to the filtrate, the basic substances being entirely inactive, even in high concentrations. From the 80 g. of original substance, 60·8 g. remained in the filtrate. The usual growth tests gave the following results:

Table III.

Medium	Dry weight of mycelium g.
Basal + 0·075 g. substance from phosphotungstic acid filtrate	0·245
Basal + 0·1 g. substance from phosphotungstic acid filtrate	0·315
Basal + 0·1 g. substance from phosphotungstic acid ppt.	—

A portion (30 g.) of the substance not precipitated by phosphotungstic acid was dissolved in 200 cc. of water, and the liquid was saturated with pure barium hydroxide; 3 volumes of alcohol were then added, and the mixture was kept at 0° overnight. The precipitate was washed with 75 % alcohol, and the precipitate and filtrate fractions were decomposed separately by means of sulphuric acid. The material recovered from the precipitate amounted to 21·6 g., and from the filtrate 5·6 g. The loss of material thus amounted to 2·8 g., and the proportion of precipitate fraction to filtrate fraction was almost exactly 4 : 1. Media were prepared containing the appropriate amounts of the two fractions and growth tested (Table IV). Since, as shown in the table, the activity had apparently been almost entirely lost, the usual toxicity tests were applied, but no evidence of the presence of any toxic substance could be obtained. Portions of the two fractions were then recombined, to reconstitute the original substance, when full activity was found to have been restored.

Table IV.

Medium	Dry weight of mycelium g.
Basal + 0·08 g. Ba ppt. fraction	0·02
Basal + 0·02 g. Ba filtrate fraction	0·018
Basal + 0·08 g. ppt. + 0·02 g. filtrate	0·285
Basal + 0·07 g. ppt. + 0·03 g. filtrate	0·265

These results gave a clear indication that the active substance present in the lentil concentrate could be fractionated into two substances, either of which alone was inactive, but in combination possessing the full activity of the original concentrate. By submitting the two fractions again to the precipitation, the activity of the separate fractions was further reduced, the amount of mycelium obtained being too small to allow of its being scraped from the surface of the medium. The process was repeated with a further small batch of the lentil concentrate (6 g.) and a similar result again obtained.

At this point it was realised that the results so far obtained—particularly the partition of the active factor into two inactive components—showed a close parallel with those reported by several observers on the "bios" of yeast. Eastcott [1928] claimed to have obtained two such fractions from a bios concentrate, and further stated that one of the factors was *i*-inositol (ppt. fraction). Although many subsequent workers had disputed her results [Narayanan, 1930] it was thought worth while to test the effect of inositol on the growth of *N. gossypii* in the presence of the baryta filtrate fraction. (Inositol alone had been found inactive in promoting growth—see above, p. 1659.) The results are given in Table V.

Table V.

Medium	Dry weight of mycelium g.
Basal + 0·02 g. Ba filtrate + 0·0005 g. inositol	0·13
" " " + 0·001 g. "	0·15
" " " + 0·005 g. "	0·19
" " " + 0·007 g. "	0·245
" " " + 0·01 g. "	0·315

Other media were tested in which the inositol was replaced, wholly or in part, by mannitol, but in no case was the presence of mannitol found to influence the amount of growth. The inositol itself was also carefully recrystallised several times, but again the repurified product did not differ in activity from the original sample (Kahlbaum). The evidence seemed conclusive that small amounts of inositol were able to replace the "baryta precipitate" fraction in the medium, in conjunction with the "baryta filtrate."

For this reason, the isolation of inositol from the baryta precipitate fraction was attempted. 20 g. of the fraction were dissolved in water, and the solution was treated with cold neutral lead acetate; after removal of the small precipitate, the liquid was treated with excess of basic lead acetate and ammonia, and allowed to stand overnight. The precipitate was removed, and decomposed with hydrogen sulphide in the presence of sulphuric acid; from the solution,

excess of sulphuric acid was quantitatively removed, and the liquid was concentrated to a small bulk. Five volumes of absolute alcohol and 1 volume of ether were added, and the whole was kept at 0° for 24 hours. The solid was removed and taken up in as little hot water (containing about 5 % nitric acid) as possible, and reprecipitated with alcohol and ether. After again taking up in hot water, the solution was treated with hot baryta solution; the filtrate was freed from barium with ammonium carbonate, evaporated to a small bulk, and precipitated with alcohol and ether. The precipitate was recrystallised from 50 % acetic acid, and after three recrystallisations was obtained as a white crystalline material, M.P. 223°. This contained[1] C, 40·4 %; H, 6·9 %. Calculated for inositol, $(CHOH)_6$, C, 40·0 %; H, 6·7 %. A mixed M.P. with a sample of Kahlbaum's inositol, recrystallised three times, showed no depression.

The hexa-acetyl derivative, prepared by the method of Müller [1907], had M.P. 214°; it showed no depression when mixed with a sample of hexa-acetyl-inositol prepared from Kahlbaum's inositol. It was therefore considered that the product isolated from the baryta precipitate fraction was inositol, which was known to be widely distributed among natural products. The amount isolated was 0·88 g. from 20 g. of the baryta precipitate fraction. A calculation of the amount present, based upon the relative activities of pure inositol and the precipitate fraction indicated the presence of about 2 g. of inositol in 20 g. of the fraction. The yield thus amounted to approximately 40 %, which was considered reasonable in view of the complexity of the process of separation.

Its growth-promoting activity was tested in the usual way, and was found to be the same as that of Kahlbaum inositol. 0·007 g. in conjunction with 0·02 g. of the baryta filtrate fraction in 50 cc. of medium gave 0·235 g. of mycelium (dry weight). The remainder of the baryta precipitate fraction, from which the inositol had been removed by the process described above, was carefully freed from lead, acetic acid, *etc.*, and portions tested for activity. It was found to be completely inactive and non-toxic, thus proving that the active factor had been entirely removed by precipitation with basic lead acetate. An experiment with pure inositol had shown that this substance was completely separated from a mixture with sugars by the same method. There seemed no doubt, therefore, that the substance responsible for the activity of the baryta precipitate fraction was inositol, and that no other active substance was present.

The baryta filtrate fraction.

So far, little has been done in attempts to isolate the active substance from the baryta filtrate fraction. Precipitation with the mercuric acetate reagent of Neuberg and Kerb was found to throw down the active substance, so that the possibility exists that it is associated with the amino-acids. That it is not one of the ordinarily occurring amino-acids follows from the fact that the hydrolysis mixture obtained from gelatin, supplemented with tryptophan,

[1] The micro-combustions were carried out by Dr J. A. B. Smith, to whom our thanks are due.

tyrosine and inositol was found unable to support the growth of *N. gossypii*. Investigations on this section of the work are still in progress.

Fractionation of concentrates from other sources.

The fractionation of the active factor into two components by means of baryta and alcohol in the case of lentils made it desirable to ascertain whether a similar result could be obtained when using concentrates prepared from other sources. Consequently, the following substances were investigated: peptone, tryptic digest of ox-heart, lupin extract. The method used was precisely the same as in the case of the lentil preparation, and the results obtained are given in Table VI.

Table VI.

Medium	Dry weight of mycelium g.
Basal + 0·12 g. Ba ppt. from peptone	—
„ + 0·27 g. Ba filtrate from peptone	—
„ + 0·12 g. ppt. + 0·27 g. filtrate from peptone ...	0·185
„ + 0·06 g. Ba ppt. from ox-heart	—
„ + 0·24 g. Ba filtrate from ox-heart	0·055
„ + 0·06 g. ppt. + 0·24 g. filtrate from ox-heart ...	0·195
„ + 0·07 g. Ba ppt. from lupin extract	—
„ + 0·03 g. Ba filtrate from lupin extract	—
„ + 0·07 g. ppt. + 0·03 g. filtrate from lupin extract	0·155

The results clearly indicated that the active factor present in each of these substances was split into two components, neither of which alone was active, but which in conjunction gave the normal degree of activity.

In the case of the lupin extract, inositol was actually isolated from the baryta precipitate fraction, the method being the same as that used in the experiments with lentils. The inositol-free residue was again shown to be inactive in combination with the baryta filtrate fraction.

Various experiments on the baryta precipitate fraction (inositol).

(*a*) *Hydrolysis with 25 % sulphuric acid.* Eastcott [1928] found that the "bios I" (inositol) content of her preparation, as judged by its "bios" activity, was trebled on boiling with 22 % hydrochloric acid for 24 hours. This she ascribed to the presence of a precursor of inositol, from which the latter was liberated by hydrolysis with acid. That no similar effect could be observed in the present case was proved by the following experiment. 25 g. of the lentil concentrate, after treatment with phosphotungstic acid, were hydrolysed with 25 % sulphuric acid for 24 hours. A large amount (3·2 g.) of insoluble matter separated. The solution after hydrolysis was freed from sulphuric acid in the usual manner, and the filtrates were evaporated. 15·8 g. of solid material were recovered, so that there was a loss of 6 g.; the loss was probably partly due to adsorption on the barium sulphate precipitates, and partly to destruction of sugars in the concentrate by the action of the strong acid. The activity of the product after hydrolysis was tested as usual (Table VII).

Table VII.

Medium	Dry weight of mycelium g.
Basal + 0·025 g. concentrate after hydrolysis	0·11
„ + 0·05 g. „ „ „	0·165
„ + 0·075 g. „ „ „	0·255
„ + 0·075 g. original concentrate	0·245

(*b*) *Recovery of the active factor from the mycelium.* In her work on "bios," Eastcott was able to recover from the yeast-crop grown on a medium containing a known amount of inositol the whole of the inositol supplied. Further, many workers have apparently shown that yeast manufactures "bios" during growth. It seemed therefore of interest to determine whether any similar effect could be traced in the case of *N. gossypii*. Extracts were prepared from the combined mycelia from 2, 4, 6, and 8 plates, and from the residual media after removal of the mycelia. Fresh media were prepared incorporating these extracts, allowance being made for both the nitrogen and the phosphate carried over in the extracts. In no case was the medium found able to support more than slight growth. If a large amount of extract of mycelium were used (1 g. in 50 cc. medium), the growth amounted only to 0·185 g. The evidence was, therefore, that far from producing the active factors during growth, the fungus largely used up the amount initially supplied in the medium. These results were thus in direct contrast to those noted by workers in the case of yeast and "bios."

(*c*) *Proportion of inositol to the baryta filtrate fraction giving optimum growth.* Since it had been proved that the accessory factor in the case of *N. gossypii* consisted actually of two substances, it seemed probable that the activity of an extract would depend not only on the total amount of the accessory factor present, but on the relative amounts of the two components, and that the high activity of such products as the lentil concentrate might be due to the presence of the two components in an optimum ratio. Experiments were made to see

Table VIII.

Medium			Dry weight of mycelium g.
Basal + 0·01 g.	lentil extract	... ...	—
„	„	+ 0·001 g. inositol	0·065
„	„	+ 0·005 g. „	0·07
„ + 0·025 g.	„	... ...	0·06
„	„	+ 0·001 g. inositol	0·085
„	„	+ 0·005 g. „	0·09
„ + 0·05 g.	„	... ...	0·12
„	„	+ 0·001 g. inositol	0·185
„	„	+ 0·005 g. „	0·195
„ + 0·075 g.	„	... ...	0·195
„	„	+ 0·001 g. inositol	0·21
„	„	+ 0·005 g. „	0·23
„ + 0·10 g.	„	... ...	0·28
„	„	+ 0·001 g. inositol	0·296
„	„	+ 0·005 g. „	0·30
„ + 0·01 g.	„	+ 0·01 g. inositol	0·08
„ + 0·05 g.	„	„ „	0·27
„ + 0·075 g.	„	„ „	0·29
„ + 0·10 g.	„	„ „	0·305

whether the addition of extra inositol to the lentil preparation gave any increased activity (Table VIII).

These results indicated that the activity of the lentil extract was somewhat increased by the addition of small amounts of inositol, the effect being most noticeable when dealing with small amounts of the concentrate. Relatively much less effect was observed when the medium contained larger amounts of the lentil preparation, suggesting that the fungus was unable to utilise amounts of inositol beyond a certain concentration.

The effect of adding inositol to marmite was also tested. As shown above (p. 1660), marmite was not found to be a particularly rich source of the accessory factor, but when supplemented with inositol, its activity was considerably increased. The conclusion was drawn that marmite was rich in the second factor but deficient in inositol.

Table IX.

Medium	Dry weight of mycelium g.
Basal + 0·075 g. marmite	0·11
„ „ „ + 0·01 g. inositol	0·24
„ + 0·15 g. marmite	0·185
„ „ „ + 0·01 g. inositol	0·31

(*d*) *Activity of derivatives of inositol.* In an attempt to ascertain whether the activity of inositol could be due to the presence of small amounts of active impurities, media were tested in which portions of the hexa-acetylinositol, prepared as described above, were incorporated. The derivative itself is insoluble in water, but an active impurity, unless itself converted into an insoluble acetyl compound, would be soluble and removed during acetylation. Further, the inositol obtained by hydrolysis of the acetyl compound should recover the full initial activity of the original inositol. On determining the activity of the derivative, it was found that the media containing the hexa-acetyl inositol supported a certain amount of growth (Table X). On further examining

Table X.

Medium	Dry weight of mycelium g.
Basal + 0·03 g. baryta filtrate + 0·025 g. hexacetyl inositol	0·225
Basal + 0·03 g. baryta filtrate + 0·007 g. inositol derived from hexacetyl compound...	0·285

this point, it was found that the acetyl compound was partly hydrolysed during the autoclaving of the medium; a loss of about 10 % occurred by autoclaving with water alone, and a hydrolysis amounting to some 80 % in the presence of small quantities of potassium phosphate. Thus the activity shown by the hexa-acetylinositol could be regarded as due to the liberation of free inositol in the medium. A test using a sample of inositol prepared from the hexa-acetyl derivative by hydrolysis gave the same amount of growth as given by the original inositol. If the activity of inositol were due actually to the presence

of an impurity, this impurity must be inseparable from inositol by acetylation, and the amount present too small to influence the ordinary chemical and physical properties of inositol and its hexa-acetate.

Discussion.

The experiments described above seemed to show conclusively that the accessory factor necessary for the growth of *N. gossypii* could be fractionated into two mutually complementary substances, one of which was shown to be inositol. The nature of the second factor was not determined; it was found to be precipitated with the monoamino-acids by Neuberg and Kerb's reagent, but it was not one of the amino-acids normally occurring among protein hydrolysis mixtures. Whether it was a single substance was not determined, but there was no evidence to the contrary.

What part is played by inositol in the metabolism of the fungus is entirely unknown. It is curious that this substance has been stated repeatedly to have some special significance in the promotion of growth or activity, and its isolation as a constituent of "bios" has been reported [Eastcott, 1928]. The view that inositol is concerned in "bios" activity is, however, by no means generally accepted. Thus Narayanan [1930], criticising the work of Eastcott, stated that "the activity of inositol must have been due either to the presence of an impurity, or to some such effect as has been described by Reader in the case of mannitol." (The following communication contains a further discussion of this point.)

In her observations on the effect of mannitol on bacterial growth, Reader [1929] used a medium in which 0·5 % glucose constituted the source of carbohydrate. To this was added a small amount (1/20 "unit dose" per 20 cc.) of torulin, and 0·5 % of mannitol. The resultant growth of *S. corallinus* showed a large increase (from 300 % to 600 %) over that given in the absence of mannitol. A similar effect, though much less pronounced, was given by citrate. When the concentrations of torulin and mannitol were reduced to 1/400th of their original amount, the excess growth due to mannitol was not observed. The explanation of these "anomalous results" put forward by Reader was that mannitol served as a "specific source" of carbohydrate food-supply for her organism. Since Eastcott had found that the addition of inositol to "bios II" gave an increased growth of yeast amounting to about 300 %, Reader considered that this result could similarly be explained on the grounds that inositol served as a "specific source" of carbohydrate for yeast.

This interpretation does not seem applicable to the results here described for the following reasons. Reader, using a medium containing glucose and torulin (no mannitol), obtained a reasonably good growth of her organism, under the standard conditions adopted; similarly, Eastcott, using a medium containing "bios II" without inositol obtained a 20-fold increase of yeast cells in 24 hours. On the other hand, *N. gossypii* does not give any measurable

growth even in the presence of large amounts of the baryta filtrate fraction, which corresponds to Eastcott's "bios II"; growth is of the starvation type, the surface of the medium being faintly clouded, as in the case of media containing gelatin only. It has been found possible to remove and weigh crops of mycelium amounting to 0·008 g. (dry weight, from 2 plates), and the growth of starvation type probably does not amount to more than 2–3 mg. dry weight per 2 plates. The addition of mannitol to the medium used by Reader gave a 5- or 6-fold increase of growth of *S. corallinus*; the addition of inositol to the medium in the case of *N. gossypii* resulted in a far greater increase of growth—at least 30 times. Further, the amount of mannitol used by Reader was at least 10 % of the amount of glucose in the medium, whereas in the present case active growth was produced when the inositol present was only 0·6 % of the glucose. None of the results given by Reader refers to the use of mannitol in such extreme dilution, except when torulin (corresponding to the baryta alcohol filtrate here) was also diluted. The conditions of the experiments here described seem to correspond most nearly to those in which Reader used the diluted solutions of mannitol and torulin, and in which she did not obtain "anomalous results." The evidence here is that inositol is a true growth-promoting factor, essential for healthy growth, and not merely a "specific food factor."

In this connection, an estimation was made of the amount of glucose taken from the medium during the growth of the fungus, by determining the reducing sugar present in 50 cc. of medium before and after growing the fungus thereon. It was found that a crop of the fungus, dry weight 0·28 g., had used 370 mg. of glucose during growth, the amount of inositol supplied in the medium being 8 mg. The small amount of inositol which could have been used, in comparison with the amount of glucose actually used, gave further support to the contention that inositol is more than a "specific food factor."

If the activity of inositol is to be explained on the basis of the presence of an impurity, the amount of impurity must be too small to be detected by the ordinary tests for purity, or removed by the chemical methods usually available.

Summary.

1. The statement of Farries and Bell, that *Nematospora gossypii* is unable to assimilate simple nitrogenous compounds in synthetic media, unless supplied with small amounts of an "accessory factor," is confirmed.

2. The accessory factor is shown to be present in association with crude proteins from various sources, and in extracts of lentils and etiolated lupin seedlings; cotton leaf extracts, potato, and yeast extracts show less activity.

3. The accessory factor yields two components on precipitation with barium hydroxide and alcohol; neither alone is active, the presence of both fractions being necessary.

4. The active substance in the fraction precipitated by barium hydroxide and alcohol can be replaced in the medium by small amounts of inositol.

5. The active substance present in the fraction not precipitated by barium hydroxide and alcohol is precipitated by mercuric acetate and sodium carbonate.

6. Inositol has been isolated from the baryta precipitate fraction; the fraction after removal of the inositol is inactive.

7. The activity of some relatively inactive substances (*e.g.* marmite) is greatly increased by the addition of inositol; such substances are therefore considered to contain the "baryta filtrate" fraction, and to owe their lack of activity to a deficiency of inositol.

8. Inositol is not considered to act as a "specific food factor," as is the case with mannitol in the growth of *S. corallinus* (Reader).

We wish especially to record our indebtedness to Prof. W. Brown, with whom the general research on the nutritional requirements of fungi originated, and at whose suggestion we undertook the more purely chemical investigations involved, and to Prof. A. C. Chibnall for much valuable advice and criticism.

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CLXXX. THE ACCESSORY FACTOR NECESSARY FOR THE GROWTH OF *NEMATOSPORA GOSSYPII*.

II. THE RELATION OF THE ACCESSORY FACTOR TO "BIOS."

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In view of the results recorded in the preceding communication it obviously became desirable to determine whether preparations known to possess "bios" activity towards yeast could promote the growth of *N. gossypii*, and, on the other hand, whether concentrates known to show activity in respect of this fungus could serve as a source of "bios."

A study of the literature regarding "bios" shows that there is still far from general agreement as to the nature of this substance, and even as to its existence. With regard to the latter point, the work of Copping [1929] seems to summarise the present position. As the result of a survey of the nutritional needs of a very large number of yeasts, as well as other organisms, she concluded that the yeasts differ widely in their requirements; some varieties she found were able to reproduce indefinitely on synthetic media, while others, including *S. cerevisiae*, were unable to grow normally unless supplied with a factor of the "bios" type.

The chemical nature of "bios" has been and still is a matter of considerable dispute; extremely active concentrates have been prepared, without throwing any light on the identity of the substance. Claims to have separated "bios" as a chemical entity have from time to time been made, but these results have not found general acceptance. The crystalline substance isolated by Eddy *et al.* [1924], and claimed to be a pure "bios," was found to be less active than a concentrate prepared by Narayanan, and admitted to be a mixture. The concentrate of Narayanan seems to be the most active preparation yet recorded, despite its admitted impurity.

The apparent similarity of the results recorded in the preceding paper and those reported by Eastcott [1928], made it desirable to examine further the relation between the active substances necessary for the growth of *N. gossypii* and of yeast. The results indicated definitely that the two factors are not identical.

Experimental.

(1) *"Bios" activity of concentrates containing the accessory factor for* N. gossypii.

The technique adopted in the experiments on the growth of yeast was that described by Narayanan [1930]. The medium used was that of Reader [1927], which was shown by Copping [1929] to be satisfactory. The strain of yeast employed was subcultured on wort-agar slopes, and cultures of 24–30 hours' growth were used for the tests. The inoculations of the sterile Reader's medium were so made that the original yeast-count of the culture was 2500 cells per cc. Adopting the system of Narayanan, this is shown in the table as a "count" of 0·01. The cultures were incubated at 23°, and a count was made after 30 hours; a multiplication of over a hundredfold in the number of cells within this period was taken to indicate "bios" activity.

The substances tested and the results obtained are summarised in Table I. The baryta-alcohol filtrate was prepared from lentil extract after removal of the substances precipitable by phosphotungstic acid, as described in the preceding communication.

Table I.

Substance tested	Amount per 25 cc. Reader's medium (g.)	Initial count	Count after 30 hrs.
Control (medium alone)...	—	0·01	0·01
Lentil concentrate	0·001	0·01	1
" "	0·005	0·01	2
Baryta-alcohol filtrate	0·001	0·01	0·01
" "	0·005	0·01	0·02
Inositol	0·005	0·01	0·05
Baryta-alcohol filtrate *plus* inositol ...	0·003 + 0·005	0·01	0·13
Marmite	0·005	0·01	0·01 (?)
"	0·010	0·01	1·3
"	0·015	0·01	3

It appeared from these results that the lentil concentrates possessed considerable "bios" activity, and were in fact rather richer in "bios" than yeast preparations (marmite). The two main fractions prepared from the lentil concentrate, inositol and the baryta-alcohol filtrate, were both devoid of activity, and the activity was not restored on re-combining them. This seemed curious, and indicated that the "bios" substance of the lentils had been lost at some stage of the fractionation. The loss may have occurred at the phosphotungstic acid stage, since Narayanan states that "bios" is precipitated by this reagent (although the majority of other workers do not find this to be the case). Another possibility was that the baryta-alcohol filtrate fraction contained traces of inhibitory substances; there was at any rate no substance present toxic to *N. gossypii*, and the question was not pursued further. It may be noted that inositol showed no activity in promoting yeast growth, this observation agreeing with those of Narayanan. The growth produced in the

presence of inositol amounted approximately to a fivefold increase, *i.e.* to only one-twentieth of that considered as evidence of true "bios" activity. The contention of Reader and of Narayanan, that this effect is parallel with that of mannitol on the growth of *S. corallinus*, seems plausible.

(2) *Growth of* N. gossypii *in the presence of substances of known "bios" activity.*

It has been shown in the preceding paper that marmite, a product of recognised "bios" activity, is a comparatively poor source of the accessory factor for *N. gossypii*; and further, that this lack of activity is due to a deficiency of inositol. Through the kindness of Prof. J. C. Drummond, we were able to test the effect of an extremely active sample of "bios" on the growth of the fungus. This material had been prepared from yeast and was obtained as a solution containing 0·6 % of organic matter, active in promoting a vigorous growth of yeast in doses of 0·2 cc. per 2 cc. salt-glucose medium (*i.e.* 0·0012 g. solid organic matter per cc.).

When tested in the usual way for the *Nematospora* factor, the preparation was found to be entirely inactive in doses up to 0·05 g. organic matter per 50 cc. medium. That no toxic substance was present was shown by the usual tests. Since the preparation of the "bios" concentrate had involved precipitation with basic lead acetate, any inositol originally present would have been removed at this stage; to this extent, therefore, the preparation corresponded to the baryta-alcohol filtrate fraction of the lentil concentrates, and its lack of activity for *N. gossypii* was to be expected. There still remained the possibility that this "bios" preparation contained the second *Nematospora* factor, *i.e.* would give growth of the fungus when in the presence of inositol. Unfortunately, lack of material made it impossible to test this point. Recent work on other lines has given definite indications that this is not the case, and that although concentrates of the accessory factors for *N. gossypii* may show "bios" activity, the factor responsible is quite distinct from those promoting growth of the fungus.

Summary.

The growth-promoting accessory necessary for *N. gossypii* is found in association with "bios" among natural products, but the two substances are not identical. Inositol is devoid of true "bios" activity, but is essential for the growth of *Nematospora*.

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CLXXXI. THE RÔLE OF CERTAIN DIETARY FACTORS IN THE FORMATION OF ERYTHROCYTES.

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(*Received August 17th, 1931.*)

Our knowledge about the precursors and catalysts involved in the production of red blood cells is very meagre. The most important advances in this field during recent years have been the recognition of copper as an important element concerned in the formation of haemoglobin [Hart *et al.* 1928] and of the principles present in liver, which are curative of pernicious anaemia [Minot and Murphy, 1926] which are now considered to be a peptide composed of β-hydroxyglutamic acid and l-γ-hydroxyproline [Dakin, West and Howe, 1931] and the more recently discovered tribasic acid of the composition $C_{11}H_{15}O_7N$, H_2O which gives reactions of the pyrrole group [Dakin and West, 1931]. The observation that a commercial liver extract (Eli Lilly and Co., No. 343) was a very potent source of vitamin B_2 and cured symptoms of depilation in rats [Guha, 1931, 1, 2] and was at the same time a product highly effective in pernicious anaemia raised the question of a possible relationship between the two factors concerned. While the possibility of a chemical identity was excluded on several grounds [Guha, 1931, 2, 3], it was considered of interest to investigate whether vitamin B_2 had a similar haematopoietic effect. The recent observations of Bliss [1930] and of Bliss and Thomason [1931] on the relation of pellagra to the blood picture, lent further point to this question. The rôle of vitamin B_2 in the economy of the organism is at present not understood. Hassan and Drummond [1927] observed that a high protein diet required a larger allowance of the relatively heat-stable component of marmite, which was presumably vitamin B_2, for the good growth of rats. Kon [1929] observed an increase in the urinary C/N ratio in vitamin B_2 deficiency. But no conclusive evidence was obtained by Kon [1931] to show that vitamin B_2 has a specific influence on nitrogen metabolism. Leader's work [Leader, 1930], on the other hand, tends to show that sugar in the diet has a predisposing effect towards the production of experimental pellagra. In fact, although a considerable number of attempts have been made to correlate one or other of the vitamins with processes of general metabolism, such studies cannot be said to have yielded so far any significant results. It

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was desirable, therefore, to attempt to explore the mode of action of vitamin B_2 in a different direction.

While this work was in progress, Sure, Kik and Smith [1931] published a note, in which they reported a diminution in haemoglobin concentration and in the red blood cell count in vitamin B_2 deficiency, when it was accompanied by skin lesions. This type of anaemia was not observed in deficiency of vitamins A, D or E and it was considered that the secondary anaemias sometimes observed in keratomalacia in man were probably due to secondary disorders and not specifically to vitamin A deficiency [Sure, Kik and Walker, 1929, 2; Sure, Kik and Walker, 1931; Sure and Kik, 1931]. Sure, Kik and Walker [1929, 1] had, however, formerly observed fluctuations in the concentration of erythrocytes and haemoglobin in rats on diets deficient in the vitamin B complex. These results appear to indicate a certain degree of specificity in the diminution of the erythrocyte concentration in vitamin B_2 deficiency.

The effect of minute amounts of copper in nutritional anaemia has been shown by Hart *et al.* [1928]. While, however, these authors found no evidence for the existence of an organic substance necessary for haemoglobin regeneration in rats on a whole milk diet, Drabkin and Miller [1931] have recently reported the efficacy of certain amino-acids, glutamic acid and arginine in particular, in cases of nutritional anaemia in the rat. In view of these statements and of the suggestion of Bliss [1930] that pellagra might be due to simple iron deficiency, the combined effect of copper, iron and glutamic acid on vitamin B_2-deficient rats has been studied. The effect of vitamin B_1 deficiency has also been studied for purposes of comparison.

It has been recorded before [Guha, 1931, 2] that an aqueous extract of liver suffers a great loss in vitamin B_2 on being autoclaved in an alkaline medium. As one of us (L. W. M.) has found, in course of an investigation shortly to be published on the rôle of liver in normal nutrition, that the feeding of fresh liver produces a marked increase in the red cell count of normal rats, it was of interest to enquire whether the feeding of an aqueous liver extract, in which vitamin B_2 was destroyed by autoclaving, was capable of raising the erythrocyte count of vitamin B_2-deficient animals. The results obtained indicate the existence of a heat-stable factor in liver, which has a most pronounced effect on the formation of red blood cells. That it is probably different from the factor responsible for the cure of pernicious anaemia appears from considerations, which will be discussed later.

The present work, which was begun to investigate the relation, if any, between vitamin B_2 and the erythrocyte count, has thus been extended to cover the following points.

1. The effect of vitamin B_2 deficiency, followed by administration of vitamin B_2, on the red cell count.

2. The influence of copper, iron and glutamic acid, if any, on the erythrocyte count in vitamin B_2 deficiency.

3. The question of the iron intake in vitamin B_2 deficiency.
4. Other dietary factors concerned in the production of erythrocytes.
5. The effect of vitamin B_1 deficiency on the red cell count.

Experimental.

Both albino and piebald rats, raised in this laboratory, were used. Blood for red cell counts was taken after clipping the end of the tail, and counts were taken with a Bürker haemocytometer. Haldane's method was used for estimations of haemoglobin, but as enough blood was not always obtainable for accurate estimations, these were carried out only in a small number of cases. Whether the haemoglobin concentration goes hand in hand with the red cell count or not, will, therefore, have to be settled by further work, which is in progress.

The following basal diets were fed in these experiments.

Diet 16	%	Diet 16 (*a*)	%
Rice starch	65	Rice starch	40
Palm kernel oil	10	Cane sugar	17
"Light white casein"	21	"Light white casein"	23
Salt mixture (Osborne and Mendel)	4	Palm kernel oil	15
		Salt mixture (Osborne and Mendel)	5

Diet 16 (*b*) was made up by incorporating 12 g. dried yeast per 100 g. of diet 16.
Diet 16 (*c*) was made up by incorporating 10 g. dried yeast per 100 g. of diet 16 (*a*).

All the animals received 2 drops of cod-liver oil daily. All other supplements were also fed separately from the basal diet unless otherwise stated. The vitamin B_1 preparation was obtained by the extraction of brewer's top yeast with boiling water and precipitation with neutral lead acetate, followed by adsorption with and elution from fuller's earth [Guha, 1931, 4]. Such a preparation provides an excellent source of vitamin B_1, practically free from vitamin B_2 [Guha, 1931, 2]. The vitamin B_2 preparation was obtained in the usual way by autoclaving marmite with baryta at p_H 9 for $1\frac{1}{2}$ hours at 18 lbs. pressure. Such a preparation, while it is a rich source of vitamin B_2, contains negligible quantities of vitamin B_1 [Guha, 1931, 2, 4]. The aqueous liver extract, free from vitamin B_2 was obtained by a modification of the method described before [Guha, 1931, 2]. Fresh ox-liver (100 g.) was finely minced, suspended in distilled water (300 cc.), brought to p_H 5 with dilute HCl, and boiled up for 5 minutes. The filtrate was brought to p_H 9 with dilute NaOH and autoclaved for $1\frac{1}{2}$–3 hours at 18 lbs. pressure. The resulting preparation (3 cc. = 1 g. of fresh liver) was almost entirely devoid of vitamin B_2 (Fig. 1).

The copper sulphate, ferric chloride and glutamic acid were administered in daily doses of 0·1 cc., 0·5 cc. and 1 cc. of aqueous solutions containing respectively 0·05 mg. copper sulphate, 0·5 mg. ferric chloride and 50 mg. glutamic acid.

The figures for the red blood cell count are given throughout in millions per mm.^{3} The leucocyte counts of a few vitamin B_2-deficient rats were compared

with those of normal animals but no marked differences were observed [see also Sure, Kik and Smith, 1931].

A. In these experiments animals, which had been previously undergoing partial vitamin B_2 deficiency, were subjected to relatively short periods of complete vitamin B_2 deficiency (3–4 weeks) and then their red cell counts

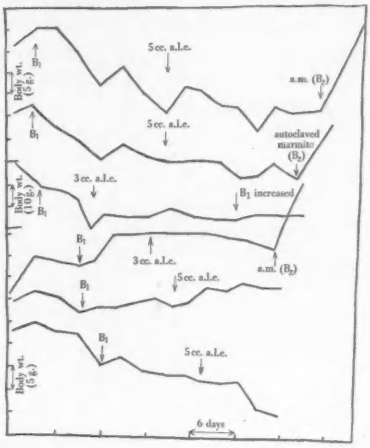

Fig. 1. Curves illustrating the ineffectiveness of autoclaved liver extract to supply vitamin B_2.
a.l.e. = autoclaved liver extract. a.m. = autoclaved marmite.

were taken. They were receiving throughout 1 cc. of the vitamin B_1 preparation, which was found from separate experiments to be ample. It was considered that the physiological rôle of vitamin B_2 should be more apparent under these circumstances than under drastic deprivation of vitamin B_2, as in this latter case secondary complications might mask the primary effect. The results are given in Table I.

Table I.

Rat No.	Colour	Sex	Age (weeks)	Period of complete vitamin B_2 deficiency (days)	Body wt. at the time of taking R.B.C. (g.)	R.B.C.	Average R.B.C.
106	Black and white	♂	25	25	129	8·0	
107	,,	♂	25	30	117	7·7	
108	,,	♂	25	30	129	7·8	
109	,,	♂	25	30	125	7·0	7·78
155	Albino	♀	20	30	105	7·9	
156	,,	♀	20	25	92	8·3	
157	,,	♀	20	30	112	7·5	
160	,,	♀	20	25	109	8·0	

The figures for normal rats, subsisting on milk, brown bread and whole wheat, of approximately the same age were 9·2, 9·2, 9·2 and 9·4, giving an

average of 9·25 millions/mm.^{3} The reduction in the red cell count in vitamin B_2 deficiency was thus relatively small but distinct, although symptoms of depilation or dermatitis had not yet supervened in these animals.

B. In these experiments, the effect of copper, iron, glutamic acid and autoclaved marmite on vitamin B_2-deficient animals was investigated. The vitamin B_1 preparation was fed in 1 cc. daily doses to all the rats. Copper sulphate, ferric chloride and glutamic acid, whenever fed, were administered in daily doses of 0·05 mg., 0·5 mg. and 50 mg. respectively. These three substances gave negative results. The effect of autoclaved marmite, in a daily dose equivalent to 0·5 g. of marmite, is shown in Fig. 2.

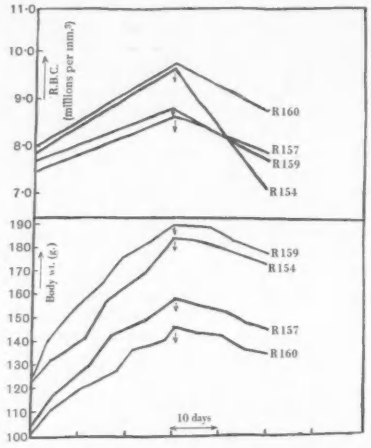

Fig. 2. Fuller's earth extract (B_1) *plus* autoclaved marmite (B_2) from beginning of exp.; ↓ autoclaved marmite withdrawn.

These results show that the diminution of the red cell count occurring in vitamin B_2 deficiency can be corrected by alkaline autoclaved marmite but not by copper, glutamic acid and additional iron.

In order to test the view of Bliss [1930], who considers vitamin B_2 deficiency as simply iron deficiency, the food intake records of the above animals were kept for 10 days, although the fact that rats Nos. 106, 155 and 156, which were receiving extra iron separately from the basal diet, showed no gain in weight, would by itself indicate that iron cannot replace vitamin B_2 for the growth of rats. The figures for the food consumption are given in Table II.

While these figures show that there is a distinct reduction in food intake in vitamin B_2 deficiency, it appears that 8·2 g. of the basal diet still carry enough iron to cover the iron requirements of the animals, as the salt mixture contained 3·2 % ferric citrate. The ineffectiveness of additional iron in the absence of vitamin B_2 also appears to confirm this view. In order to test this point further, rats Nos. 157 and 160, which were receiving autoclaved marmite,

were given restricted amounts of food (7 g. daily) for 10 days before the final erythrocyte count was taken, but in spite of this artificially lowered food consumption, they gave normal figures, which indicates that even 7 g. of the food provided a reasonable amount of iron. The vitamin B_2-deficient rats were normally consuming 8·2 g. of food daily on the average but still gave lower red cell counts.

Table II.

Rat No.	Daily food intakes for 10 days (g.)										Average daily food intake (g.)	
107	8	8	9	8	7	7	10	9	8	12	8·6	
108	6	6	9	7	7	7	10	6	10	9	7·7	8·2
109	10	8	8	8	8	9	8	8	7	9	8·3	
106	11	7	5	4	6	9	10	8	6	7	7·3	
155	4	5	6	6	8	7	8	7	6	6	6·3	6·3
156	3	5	3	6	5	6	6	7	6	6	5·3	
154	10	9	10	11	11	12	14	14	11	13	11·4	
159	7	10	11	13	11	10	12	12	12	13	11·1	10·9
157	11	10	9	11	11	11	11	12	9	11	10·6	
160	6	10	10	10	12	11	14	11	11	11	10·6	

C. The supplements administered to the animals in the above experiments were then interchanged in some cases and three of the animals were given a daily dose of 2 cc. of the autoclaved liver extract each—a preparation practically free from vitamin B_2 (Figs. 3 and 4). The striking effect of this

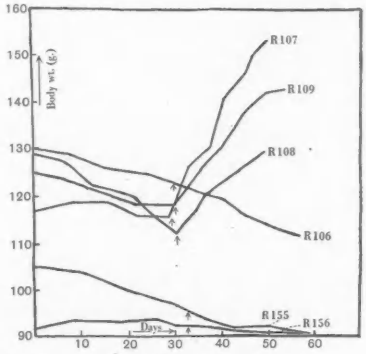

Fig. 3.

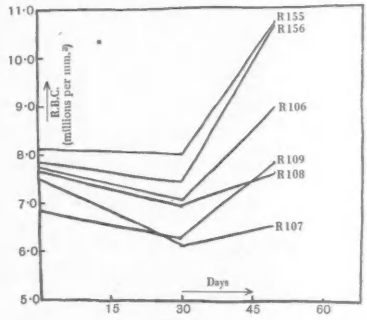

Fig. 4.

Fig. 3. R 107, R 108 and R 109, fed diet 16 + B_1, from beginning of exp.; ↑ 1 cc. autoclaved marmite in addition. R 106, R 155 and R 156, fed diet 16 + B_1 + Cu + Fe + glutamic acid from beginning of exp.; ↑ 2 cc. autoclaved liver extract in addition.

Fig. 4. Figures for red cell counts, corresponding to growth-curves shown in Fig. 3.

extract on the blood picture, in spite of the arrest of growth due to the absence of vitamin B_2 from the diet, is evident from the figures given in Table III.

It will be observed that the administration and withdrawal of autoclaved marmite were followed by a perceptible rise and fall in the red cell count. It is, however, important to notice that autoclaved liver extract, which does

not contain vitamin B_2, produces a much more marked effect than autoclaved marmite.

Table III.

Rat No.	Nature of diet	Period of feeding on diet (days)	Initial wt. (g.)	Final wt. (g.)	Initial R.B.C.	Average initial R.B.C.	Final R.B.C.	Average final R.B.C.
107	$16+B_1+B_2$	20	119	152	6·3	6·6	6·6	7·5
108	,,	20	111	128	7·1		7·8	
109	,,	20	118	141	6·4		8·0	
106	$16+B_1$ + autoclaved liver extract	20	122	113	7·2	7·7	9·1	10·3
155	,,	20	92	92	7·6		10·9	
156	,,	20	98	92	8·2		10·8	
154	$16+B_1$	20	191	181	9·8	9·3	7·1	7·6
159	,,	20	196	186	8·8		7·7	
157	,,	20	166	153	8·7		7·9	
160	,,	20	154	143	9·8		7·8	

D. Somewhat similar experiments were carried out with a group of young animals in order to compare the effects produced by (*a*) autoclaved liver extract alone, (*b*) autoclaved liver extract *plus* autoclaved marmite, and

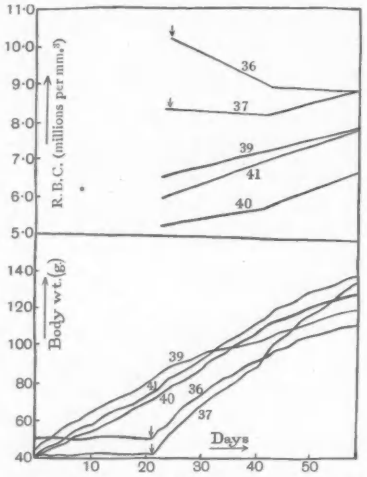

Fig. 5. Rats No. 39, 40 and 41 fed on diet 16 (*b*) throughout. Rats No. 36 and 37 fed on diet 16 + B_1 + autoclaved liver extract (3 cc.); ↓ 1 cc. autoclaved marmite in addition.

(*c*) copper, iron and glutamic acid together with a diet containing 12 % yeast (diet 16 (*c*)). The autoclaved liver extract and autoclaved marmite were fed in 3 cc. and 1 cc. daily doses respectively. In these experiments the effect of feeding the same or different supplements for different periods on the same rat was studied, the animals thus serving as their own controls. The results are given in Fig. 5.

These results indicate that the effect of 12 % yeast in the diet, supple-

mented by copper, glutamic acid and extra iron, on the red cell count, is not at all comparable with the pronounced effect of autoclaved liver extract. It also appears that the administration of autoclaved marmite in addition to autoclaved liver extract does not further stimulate the formation of erythrocytes (see also Section E). If anything it appears to have an inhibitory effect. This is in contrast to the stimulating effect of autoclaved marmite, when given by itself to rats, which had been previously undergoing vitamin B_2 deficiency (see Tables II and IV). The significance of these results is discussed later.

E. The results of experiments, similar to those described in the preceding section, on another group of rats, approximately 8 weeks old, are given in Table IV. The autoclaved liver extract and autoclaved marmite were fed in

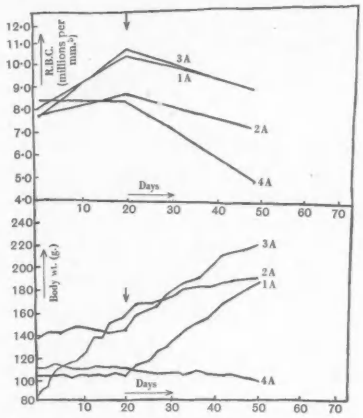

Fig. 6. 1 A and 3 A fed on diet 16 + B_1 + autoclaved liver extract; ↓ 1 cc. autoclaved marmite in addition, 2 A fed on diet 16 + B_1 + autoclaved marmite throughout, 4 A fed on diet 16 + B_1 + Cu + Fe + glutamic acid; ↓ 16 + B_1 + 0·5 cc. alkaline haematin.

3 cc. and 1 cc. daily doses respectively. In this set of experiments the effect of different supplements on the same rat was studied. In previous communications [Guha, 1931, 2, 5] it was stated that haemin, administered in a fine aqueous suspension, was unable either to restore the growth or cure the symptoms of depilation of vitamin B_2-deficient rats. As much of the haemin fed was excreted and as Kollath's experiments [1929, 1930] were carried out with alkaline haematin, 0·5 cc. of a solution of haemin in dilute sodium hydroxide containing 2·5 mg. of haemin was fed daily to one of the rats of this group with entirely negative results. As this was not taken readily it had to be incorporated in the diet. The daily administration of this alkaline solution appeared to have a deleterious effect (Fig. 6).

These results again demonstrate the quite noticeable effect of autoclaved marmite on the red cell count, while the effect of autoclaved liver extract is

very marked. The results with copper, glutamic acid and alkaline haematin are negative. The curious fact is again noticed that on feeding autoclaved marmite in addition to autoclaved liver extract, the red cell count has a tendency to fall (rats 1 A and 3 A). This confirms the experiments described in the preceding section.

Table IV.

Rat No.	Colour	Sex	Nature of diet	Period of feeding on diet (days)	Initial wt. (g.)	Final wt. (g.)	Initial R.B.C.	Final R.B.C.	Final haemoglobin concentration (g. in 100 cc. blood)
1 A	Albino	♂	$16+B_1$	25	—	107	—	8·0	—
,,	,,	,,	$16+B_1$ + autocl. liver ext.	20	107	108	8·0	10·3	—
,,	,,	,,	$16+B_1$ + autocl. liver ext. + autocl. marmite	27	108	183	10·3	8·9	14·6
2 A	,,	,,	$16+B_1$	25	—	82	—	7·7	—
,,	,,	,,	$16+B_1$ + autocl. marmite	20	82	157	7·7	8·6	—
,,	,,	,,	,,	27	157	187	8·6	7·0	14·0
3 A	,,	,,	$16+B_1$	25	—	136	—	7·6	—
	,,	,,	$16+B_1$ + autocl. liver ext.	20	136	146	7·6	10·6	—
,,	,,	,,	$16+B_1$ + autocl. liver ext. + autocl. marmite	27	146	219	10·6	8·9	15·4
4 A	Black and white	,,	$16+B_1$	25	—	113	—	8·3	—
,,	,,	,,	$16+B_1+CuSO_4+FeCl_3$ + glutamic acid	20	113	104	8·3	8·4	—
,,	,,	,,	$16+B_1$ + alk. haematin	27	104	98	8·4	4·6	10·6*

* On the day on which the last estimations were made this animal was extremely miserable and nearly dying.

F. Seven young black and white rats of the same litter, which were split up into three groups and were fed on basal diets (diets 16 (*a*) and 16 (*c*)), slightly different from the basal diets fed in the foregoing experiments, gave the following figures for the erythrocyte and haemoglobin concentrations (Table V). The autoclaved liver extract was fed in 2 cc. daily doses.

Table V.

Rat No.	Sex	Nature of diet	Period of feeding on diet (days)	Initial wt. (g.)	Final wt. (g.)	R.B.C.	Average R.B.C.	Hb (g. per 100 cc. blood)	Average Hb (g. per 100 cc. blood)
65	♀	16 (*c*)	30	67	130	7·3	7·1	12·5	13·0
66	,,	,,	30	57	117	6·9		13·5	
67	,,	16 (*a*) + B_1	30	70	89	6·4	6·35	11·2	11·3
68	,,	,,	30	80	88	6·3		11·4	
69	,,	16 (*a*) + B_1 + autocl. liver ext.	30	66	84	9·9	9·17	13·5	13·6
70	,,	,,	30	70	84	9·0		13·5	
71	,,	,,	30	60	79	8·6		13·8	

These results demonstrate that the presence of yeast in the diet (diet 16 (*c*)) gives somewhat higher figures for the red cell and haemoglobin concentrations

than when the basal diet (16 (*a*)) is supplemented only by vitamin B_1. But the addition of autoclaved liver extract to the diet gives a much higher figure for the red cell count than can be obtained with yeast, while the effect on the haemoglobin concentration is not so marked.

G. It was desirable to get a few figures for the red cell count in vitamin B_1 deficiency. Animals of approximately the same age were given synthetic diets with and without vitamin B_1 and the red cell concentrations of their peripheral blood estimated in the usual way (Table VI).

Table VI.

Rat No.	Colour	Sex	Nature of diet	Period of feeding on diet (days)	Body wt. at the time of taking R.B.C.	R.B.C.	Average R.B.C.
25	Albino	♂	$-B_1$	30	71	10·5	
27	,,	♀♂	$-B_1$	30	81	10·0	10·53
28	,,	♀	$-B_1$	30	67	11·1	
21	,,	♂	$+B_1$	30	115	9·8	
22	,,	♂♂	$+B_1$	30	130	10·4	10·33
23	,,	♂	$+B_1$	30	104	10·8	

While the number of these experiments is inadequate to justify definite conclusions, it appears that in vitamin B_1 deficiency, in contradistinction to vitamin B_2 deficiency, there is no reduction in the red cell count. Sure, Kik and Walker [1929,1] observed a slight rise in the red cell count in vitamin B_1 deficiency, which was accompanied by anhydraemia.

Discussion.

Whipple and his co-worker [1925], working chiefly with dogs, have shown in a series of important papers that certain food materials possess a very marked blood regenerative power in experimental secondary anaemia. The specific effect of copper in nutritional anaemia, observed by Hart *et al.* [1928], has been confirmed by Titus, Cave and Hughes [1928], who have also observed a specific effect of manganese. Beard and Myers [1929] have added nickel, cobalt and germanium to this list. It is necessary to stress the point that the factors, effective in one type of anaemia, may not be effective in a different type. Thus Robscheit-Robbins and Whipple [1929] found that Eli Lilly's commercial liver extract, No. 343, which is highly effective in cases of pernicious anaemia, was not nearly so effective in severe secondary anaemia in dogs. It is, therefore, not possible to say how far the factors with which we have been dealing in the present paper come into the picture of clinical and experimental anaemias.

The principle present in the aqueous extract of fresh ox-liver, which gives rise to supernormal concentration of erythrocytes in blood, even when the rats are in poor condition owing to vitamin B_2 deficiency, is apparently heat-stable. This response cannot be due to iron and copper, present in the liver extract, as these elements have been shown in this paper to be quite ineffective

in producing this unusual response when fed by themselves. The failure of glutamic acid, which, according to Drabkin and Miller [1931], is effective in nutritional anaemia in the rat, indicates that the principle in liver is of a different nature. Though we have not yet obtained conclusive evidence that this substance is different from the factors concerned in the cure of pernicious anaemia, an identity between the two appears to be improbable. Thus, as has been pointed out above, the factors curative of pernicious anaemia have been stated to be of relatively slight effect in cases of secondary anaemia, and it is, therefore, improbable that the factor in fresh liver, described in this paper, which exhibits its effect apparently even under normal conditions, is identical with them. Preliminary experiments with the E.L. liver extract No. 343 indicate that it is not nearly so effective under our conditions of experiment as autoclaved liver extract. Moreover, it would appear from the paper of Cohn, Minot, Alles and Salter [1928] that the factor curative of pernicious anaemia described there is not particularly heat-stable, likewise the acid described by Dakin and West [1931] appears to be unstable under the conditions in which the autoclaved liver extract described in this paper is made. Further work is being done in order to settle this question.

The present work indicates that there is a diminution in the red cell concentration of blood in vitamin B_2 deficiency, which can be restored to normal by feeding autoclaved marmite, though, unlike the autoclaved liver extract, this was not found to push the erythrocyte concentration beyond the normal level. While the evidence is suggestive and prompts further investigation, it cannot yet be definitely stated that the factor concerned, which is present in autoclaved marmite, is vitamin B_2. Doubt is cast on this point chiefly by the consideration that the diminished red cell count occurring in vitamin B_2 deficiency can be increased above the normal by feeding autoclaved liver extract, which is practically free from vitamin B_2. While this experiment does not by any means show that vitamin B_2 has no haematopoietic effect, it is clear that it is possible to raise the red cell count in the almost complete absence of vitamin B_2. Whether the factor present in autoclaved marmite, which restores the red cell count to normal, is vitamin B_2 or is identical with the liver principle can be settled by obtaining a preparation of vitamin B_2, which is free from the liver factor. We have not yet succeeded in this objective, though we have been able to obtain a liver preparation, which is free from vitamin B_2.

The question of the aetiology of pellagra has been discussed elsewhere [Guha, 1931, 5]. It is obvious from the experiments described in this paper, in which food intake records were taken and additional iron was fed to some of the animals, that the arrest of growth due to vitamin B_2 deficiency cannot be due simply to iron deficiency. The reduction in the red cell count, occurring in vitamin B_2 deficiency can be corrected by autoclaved marmite but not by copper, glutamic acid or additional iron. Though we are not yet certain that this principle in autoclaved marmite is vitamin B_2, there appears to be enough

suggestive evidence to warrant the possibility that vitamin B_2 may be concerned in some phase of the mobilisation of ingested iron. Bliss and Thomason [1931] point out that, according to Elvehjem and Peterson [1927], hair is the richest source of iron in the body, and it does not appear impossible that the depilation, occurring in vitamin B_2 deficiency, might be at least partly due to a failure in this phase of iron metabolism.

Certain figures quoted by us indicate that there might be a question of balance between different factors concerned in the formation of erythrocytes. Thus the rate of erythrocyte formation due to the feeding of autoclaved liver extract appeared to be inhibited by the administration of autoclaved marmite. This inhibition was, of course, associated with increased growth produced by autoclaved marmite. Funk and Lejwa [1931], in a recent paper to which our attention was drawn after this work had been completed, mention that rapid growth induces a decrease in the number of red cells, and they consider that there are factors present in foodstuffs which stimulate, and others which inhibit, blood regeneration.

It may be interesting to state that some of the vitamin B_2-deficient animals, which were receiving vitamin B_1 and autoclaved liver extract, lost fur more readily than those receiving vitamin B_1 only, suggesting that the liver principle might hasten depilation by mobilising most of the available iron for the generation of blood, when the food intake and, therefore, the iron intake is limited.

It appears from the few measurements of haemoglobin, that we have made, that the influence of autoclaved liver extract on the concentration of red cells is relatively greater than on the concentration of haemoglobin. This might indicate a more specific effect of the liver principle on the formation of the stroma of erythrocytes.

Summary.

1. Rats under vitamin B_2 deficiency show a diminution in the red cell count, which can be restored to normal by autoclaved marmite but not by copper, glutamic acid, extra iron or alkaline haematin.

2. The arrest of growth in vitamin B_2 deficiency cannot be due to simple iron deficiency, as is shown by records of food consumption. Alkaline haematin cannot replace vitamin B_2 in the diet.

3. Vitamin B_1 deficiency does not result in a reduction of the red cell count.

4. The presence of a heat-stable factor in ox-liver is demonstrated, which exerts a powerful influence on the formation of erythrocytes.

5. This factor can be extracted with boiling water and can be freed from vitamin B_2 by autoclaving in an alkaline medium. Even when growth is arrested and the animals are in poor condition owing to vitamin B_2 deficiency, the administration of this autoclaved liver extract induces a supernormal

erythrocyte count. This effect cannot be produced by iron, copper, glutamic acid and yeast.

6. The significance of these results is discussed.

We wish to express our gratitude to Sir F. G. Hopkins for his kind interest and advice. We owe much to the skilful assistance of Miss R. Leader.

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CLXXXII. A SURVEY OF ANTHOCYANINS. I.

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Although the details of the constitution and properties of the anthocyanins can only become known as the result of their isolation in substance and the study of the pure individual pigments following the lines of the pioneering researches of Willstätter and his collaborators [1913–16], yet it is also true that much can now be gleaned from an examination of the colouring matters in solution and there are many obvious advantages attaching to this procedure.

For example, the formation and occurrence of anthocyanins can be instantly recognised by inspection and the development of a method of analysis is a necessary step towards the utilisation of this property in making attempts to solve the problems of the origins and functions of these pigments. Moreover, phylogenetic investigations are often facilitated by a knowledge of the anthocyanins of the varieties, and more rapid methods than those demanding the collection of kilograms of the dried petals are urgently required.

Finally a preliminary survey should afford evidence of the existence of interesting new types of anthocyanins, and thus pave the way for extensions of the large scale investigations.

In all cases we have employed cold 1 % aqueous hydrochloric acid in order to extract the coloured materials and the best results were obtained by using fresh flowers and by examining the solutions as soon as possible following their preparation.

Colour of flowers and of the extracts—co-pigments.

The colours of the flowers or fruits themselves do not afford trustworthy indications, but whilst orange-scarlet shades indicate the occurrence of a pelargonidin derivative no blue flower-pigments are derived from pelargonidin. On the other hand not very dissimilar bluish-red *Phlox*, crimson *Dianthus* and crimson-scarlet *Linum* contain anthocyanins derived respectively from pelargonidin, cyanidin and delphinidin.

More reliance can be placed on the colour of the acid extracts provided these are observed at the boiling-point of the solution. Under these conditions the order from orange-red to blue-red is that of anthocyanins based on pelargonidin, peonidin, cyanidin, malvidin and delphinidin.

The necessity for this precaution arises from the existence in the solutions of substances (co-pigments) which intensify and modify the colour. Willstätter

and Zollinger [1916] observed that the addition of tannin to a solution of oenin chloride in dilute hydrochloric acid intensified the colour and produced a change in tone giving a much bluer red. These authors also noticed that gallic acid had a similar but weaker effect and that the ferric reactions and alkali-colour reactions were also modified; finally they commented on the fact that the effect is specific for oenin and is not observed to a comparable extent in the case of cyanin solutions.

This is true for gallotannin (although malvin is as sensitive as oenin) but co-pigments exist for all the types of anthocyanins, although they have not yet been identified. Some preliminary experiments in this field are described in the experimental section, and we note here in illustration of specificity that the glucoside of 2-hydroxyxanthone is a powerful co-pigment for cyanin but not for mecocyanin, and that the isomeric glucoside of 4-hydroxyxanthone is far less active.

The phenomenon has little or nothing to do with salt formation and occurs in the presence of a large excess of mineral acid; it is evidently the result of the formation of weak additive complexes which are dissociated at an elevated temperature or by the action of solvents.

We have reason to believe that the formation of these complexes occurs in the flowers themselves and plays a most important part in producing variations of colour. Our interest in this aspect of the subject arose from an examination of the fuchsia. The violet inner corolla gives a bluer red acid extract than that obtained from the outer bluish-red petals, and this was at first regarded as a proof that the anthocyanins are different in the two cases. We still believe that this is part of the truth, but it was noticed that the blue-red solution from the violet petals became much redder on washing with amyl alcohol. Addition of light petroleum to the separated amyl alcohol and extraction of the mixture with 0·5 % hydrochloric acid furnished an extract which, mixed with the washed anthocyanin solution, reproduced the original blue-red solution. In this case the substance in the amyl alcohol was tannin, identified as derived gallic acid. Subsequently the phenomenon was found to be very general and some of the effects are surprisingly great and cannot yet be imitated with synthetic compounds.

Willstätter and Mallison [1915] found that the rose-coloured flowers of *Pelargonium peltatum* contained pelargonin, the colouring matter of the scarlet pelargonium, and concluded that the cell-sap of the bluer red variety must be the more alkaline.

The aqueous acid extract of the rose-coloured petals is, however, also bluish-red and acquires the colour of a pure pelargonin solution only on heating; on cooling the blue shade returns. The bluish-red phlox is another striking instance of the occurrence of a pelargonin-co-pigment complex.

It seems that great changes in the colour of varieties in a species are not brought about by changes in the p_H of the cell-sap, but rather by changes in the nature of the anthocyanin, including in this term the formation of complexes

with organic substances and possibly with metals such as iron. Haas and Hill [1929] have expressed a similar view.

An apparent exception is *Primula sinensis*, in magenta and blue varieties of which Miss R. Scott-Moncrieff has found the same anthocyanins. Here, we think that one of the genetic factors concerned is a quantitative one; the magenta flowers are much the more intensely coloured and the modifying substances present in low concentrations are unable to deal with the whole amount. In the pale-coloured rather violet-shaded blue flowers the pigment remains the same, but it is all modified according to the present hypothesis. We wish to emphasise that the evidence for the theory is that changes in colour are so often found associated with a change in the anthocyanin and that we speak only of closely related varieties or species. It is agreed that changes of p_H in the cell-sap occur at different stages in the life of the plant and also in representatives of distinct genera; for example, Willstätter and Everest [1913] made it very probable that the colouring matter of the blue cornflower is the potassium salt of cyanin, whereas the rose and dahlia contain the pigment in the form of an oxonium salt with an organic acid. It is obvious also that a genetic factor for flower colour may be concerned only with the development of a co-pigment, the anthocyanin remaining unchanged in composition and concentration.

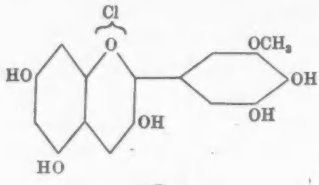
Methods of examination of the extracts and scope of the results.

The details are given in the experimental section, but in general we have observed the colour reactions with alkalis and ferric chloride and made considerable use of distributions between immiscible solvents.

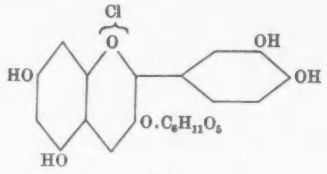
The value of this method was indicated by Schudel [1918] whose dissertation was, we understand, the result of work in Prof. Willstätter's laboratory. Willstätter and Schudel developed a technique for the separation of anthocyanidins, monoglycosidic[1] and diglycosidic anthocyanins and employed a similar process in the difficult isolation of betanin from the beet. We have made use of their observations in order to devise methods of purification of the anthocyanidins and anthocyanins and to assist in characterising the pigments.

Apart from the betanidin group and the yellow anthocyanin observed by Willstätter in the flowers of *Papaver alpinum*, it has not been found necessary to assume the existence of new anthocyanidins—we have not proved that such do not exist in the material examined, but we can accommodate our results without them. Nevertheless we are now of the opinion that petunidin chloride [Willstätter and Burdick, 1916] is essentially 3′-*O*-methyldelphinidin chloride (I) identical with the salt synthesised by Bradley, Robinson and Schwarzenbach [1930].

[1] The term "glycosidic" is employed when the nature of the sugar is unknown; "glucosidic" being used to denote a derivative of glucose.



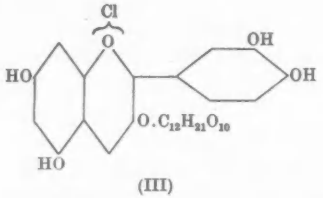
(I)



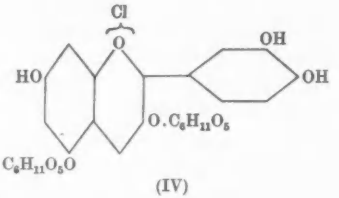
(II)

The methods we have employed throw little light on the nature of the carbohydrate groups in the anthocyanins, but in most cases we can give the position of attachment as the result of comparisons with pure natural or synthetic anthocyanins of known constitution. The simplest type of anthocyanin is represented by chrysanthemin chloride (II) which is cyanidin 3-monoglucoside.

The diglycosides can apparently be placed in one of two groups; either they are 3-biosides like mecocyanin (III) or they are 3 : 5-dimonosides like cyanin (IV). The arguments on which the constitution of cyanin are based will be fully described in papers of the series "Experiments on the Synthesis of Anthocyanins," published in the *Journal of the Chemical Society*. The evidence indicates that the 3-monosides are the basis of the anthocyanins, the other groups being subsequently attached to the molecule. Thus no 5-monoside is known. Furthermore this view affords some explanation of the changes in sugar-position type which occur, for example, in the sweet peas, the gladioli and the carnations.



(III)



(IV)

In addition, the carbohydrate residues may be acylated in the class of complex diglycosides.

Some interesting anthocyanins, not yet isolated in substance, have been encountered. The pelargonidin analogue of mecocyanin is rather widely distributed, it occurs *inter alia* in orange-red poppies, scarlet cineraria, gloxinia, tropaeolum and red-flowered anemones. Complex diglucosides of pelargonidin and delphinidin have been studied in detail by Willstätter and Mallison [1915], by Willstätter and Mieg [1915], by Karrer and Widmer [1927], and by Kondo [1931]; we have found a complex diglycoside of cyanidin in the leaves of *Coleus* and a complex diglycoside of malvidin in the flowers of *Salvia virgata nemorosa*.

Experimental.

Nature of petunidin chloride. Some comparisons of petunidin chloride with synthetic 3′-*O*-methyldelphinidin chloride were carried out by Bradley, Robinson and Schwarzenbach [1930], and no serious divergences were noted but an equimolecular mixture of delphinidin and malvidin chlorides gave almost identical results. It is remarkable that we have since found this to be true of many distribution-ratio experiments. A small specimen of finely crystallised petunidin chloride, for which we are greatly indebted to Prof. Willstätter, was available and the following experiments were carried out.

Malvidin chloride (4·002 mg.) was dissolved in 30 cc. of ethyl alcohol (1000 cc. of which contained 1 cc. concentrated hydrochloric acid) and the solution made up to 250 cc. by means of 1 % hydrochloric acid. This solution (30 cc. at 21·5°) was placed in a stoppered cylinder and shaken with air; 10 % aqueous sodium hydroxide (25 cc.) was then introduced, the liquid shaken once and immediately acidified with hydrochloric acid (10 cc.). The recovered anthocyanidin was taken up in amyl alcohol and colorimetrically estimated, using a standard prepared from malvidin chloride (0·860 mg.), ethyl alcohol (10 cc. containing hydrochloric acid as above) and amyl alcohol to 100 cc. The recovery was 78 %. Now, on performing this experiment with a solution of petunidin chloride (4·012 mg.) the recovery was quite small and indicated less than 10 % of malvidin in the sample. The recovery from both delphinidin and 3′-*O*-methyldelphinidin was less than 5 %. Using equivalent solutions of delphinidin chloride and malvidin chloride it was found that a mixture of 28 cc. of the delphinidin solution and 2 cc. of the malvidin solution gave slightly greater recovery than the natural petunidin chloride. A mixture of 7 % malvidin chloride and 93 % 3′-*O*-methyldelphinidin chloride could not be distinguished in its behaviour in the above experiment from natural petunidin. Various further tests were made with the remainder of the solutions and no divergences were noted. For example, the "93 %" and the "natural" solutions were each mixed with an equal volume of aqueous sodium acetate; the violet solutions were identical. On now shaking with 6 volumes of amyl alcohol the colour base separated as a violet film at the interface and in each case this exhibited a beautiful steel-blue reflex. In view of the facts that Willstätter and Burdick [1916] showed that petunidin yields delphinidin on demethylation and has approximately the correct methoxyl content, the only possible alternative to the methyldelphinidin hypothesis was that the substance might be an equimolecular mixture of delphinidin and malvidin. The experiments now recorded dismiss this possibility and the correctness of our conclusions was confirmed by comparisons of the extraction of equivalent solutions (50 cc.) of the "natural" petunidin chloride and of 3′-*O*-methyldelphinidin chloride (in 1 % hydrochloric acid) by successive portions (15 cc.) of the amyl ethyl ether-anisole-picric acid reagent for delphinidin mentioned below. The course of the extraction was very similar in the two cases; the pigment was gradually

removed without that sharp distinction between consecutive extracts which is characteristic of mixtures of malvidin and delphinidin. As anticipated, the first extract from the "natural" was a little more deeply coloured than that from the "synthetic"; otherwise the graded series were identical.

Tests on the original solution. The observations of the colour and of the change, if any, on heating have already been mentioned. The colour of a dilute solution of cyanin chloride is taken as standard in speaking of orange-red or blue-red.

If the colour is orange-red a small volume of the solution should be diluted with alcohol; peonidin derivatives then become much bluer and to a much greater extent than pelargonidin derivatives. In this, as in many other cases, comparison with authentic specimens is essential, and in the absence of crystallised homogeneous anthocyanins certain flower extracts will serve. For pelargonin the scarlet pelargonium; for peonin, a purified (see below) extract from the deep red peony; for cyanin, the blue cornflower; for malvin, *Epilobium* or the red-violet annual *Clarkia elegans*; for mecocyanin, any bluish-red poppy; for pelargonidin 3-diglycoside, orange-red nasturtiums; for chrysanthemin, crimson carnations; for callistephin, scarlet carnations; delphiniums and violas will give a supply of delphinidin derivatives. A portion of the original solution is shaken with amyl alcohol in order roughly to estimate the distribution number; a second extract should also be made in the case of anthocyanins of high distribution number. In the latter case, also, a test must be made for complex diglycosides. The solution is boiled in a test-tube to expel air and, whilst hot, 20 % aqueous sodium hydroxide is added drop by drop, until the colour changes through green to yellow or brownish-yellow. After actually boiling for a few seconds, concentrated hydrochloric acid is carefully added to the hot solution until the reconstituted anthocyanin indicates that the solution is acid; one more drop is then added and after 15 seconds the liquid is cooled and the distribution number again observed. In the case of monoglycosides there is no change and, although anthocyanidin is not produced under the conditions described, a second shaking is carried out for confirmation. In the case of complex diglycosides the distribution falls almost to zero.

The colour obtained on the addition of sodium acetate to the original solution is observed; this is not affected by the presence of anthoxanthins. The colours given by the pure anthocyanins are the following: callistephin, rather dull brownish violet-red; pelargonin, bright bluish-red; peonidin 3-glycosides, similar to callistephin but brighter; peonin, red-violet; cyanin, violet; mecocyanin (chrysanthemin), violet-red; malvin, bright violet; oenin, dull violet; delphinidin glycosides, blue-violet to blue. The reaction is subject to much interference, for example, from the presence of iron and/or tannin

In the case of quite clean extracts it may be desirable to test the response to the ferric reaction. Sodium carbonate is gradually added until the colour changes towards violet or blue; acid conditions are then restored by the addition of just sufficient 0·5 % hydrochloric acid. A positive reaction on the

addition of a drop of neutral ferric chloride is a deep violet coloration, replacing and more intense than the anthocyanin red. Any residual red tinge represents a negative result and the violet must be pure-toned and free from green or brown.

Examination of the anthocyanidin.

In some cases it may be necessary to purify the anthocyanin before submitting it to hydrolysis, but normally this is not the case. The solution is mixed with rather less than an equal volume of concentrated hydrochloric acid and boiled for 30 seconds (unless experiment indicates that a longer period is required). The solution is extracted with amyl alcohol and the upper layer separated and washed with water and then with 1 % hydrochloric acid. A small volume of the 1 % acid is then added in order to take up the oxonium salt after the addition of much benzene. The amount of benzene necessary to decolorise the upper layer will give some information; delphinidin requires 3–4 times the volume of the amyl alcohol; petunidin about 4–5 volumes; cyanidin, 5–6 volumes; malvidin, 5–6 volumes; peonidin, 8–9 volumes; and pelargonidin, 10–11 volumes. These figures are approximations and refer to 1 volume of the amyl alcohol solution with 3 volumes of 0·5 % hydrochloric acid. The filtered anthocyanidin solution is again extracted by amyl alcohol and the process repeated; finally, the aqueous solution of the anthocyanidin is washed three or four times with a large excess of benzene in order to remove traces of amyl alcohol.

The colour of this solution compared with that of authentic specimens gives valuable information. The following tests are carried out. A small portion is extracted with amyl alcohol and sodium acetate added; after observation a drop of ferric chloride is added and the tube gently shaken. A portion is shaken with an equal volume of a mixture of cyclohexanol (1 vol.) and toluene (5 vols.) (the cyanidin reagent); the concentration of anthocyanidin in this test should not be lower than 4 mg. in 100 cc. The upper layer is decanted into a narrow tube for observation.

A portion (concentration about 3–4 mg. in 100 cc.) is shaken with air and half its volume of 10 % sodium hydroxide added. This is immediately followed by concentrated hydrochloric acid and amyl alcohol. The recovery of anthocyanidin in this "oxidation" test is noted.

A portion (or several portions of different concentrations) is shaken with a 5 % solution of picric acid in a mixture of amyl ethyl ether (1 vol.) and anisole (4 vols.) (delphinidin reagent). (Note on recovery: amyl ethyl ether and anisole are difficult to separate by distillation, so that it is convenient to use the Abbé refractometer. The volumes % and the refractive indices are connected by a relation that is sufficiently linear for practical purposes and the following data may be of service—anisole, n, 1·5161; 4 : 1-mixture, n, 1·4902, ethyl amyl ether, n, 1·3902.)

The various anthocyanidins exhibit the following behaviour:

Pelargonidin. Amyl alcohol-sodium acetate, violet-red; no change with

ferric chloride; largely extracted by the cyanidin reagent and completely by the delphinidin reagent; not destroyed in the oxidation test. Pelargonidin is recognised by the colour of its acid solution and by the colour reactions of the anthocyanins derived from it.

Peonidin differs chiefly from pelargonidin in the colour of its acid solutions and in the colour reactions of related anthocyanins.

Cyanidin gives a reddish-violet solution when sodium acetate is added to the amyl alcohol extract over water and ferric chloride changes the violet to a bright blue colour. This is apt to be confused with malvidin containing a trace of ferric-reacting anthocyanidin. Cyanidin is fairly stable in the oxidation test; it imparts a rose-red colour to the cyanidin reagent (malvidin, a very weak mauve) and its extraction by the delphinidin reagent is not complete from dilute solutions. In order to distinguish cyanidin from impure malvidin it is best to perform the ferric reaction without sodium acetate using a carefully washed amyl alcohol solution diluted with ethyl alcohol. It is only when the anthocyanin is a 3 : 5-dimonoside that confusion with malvidin is a possibility; the reactions of cyanidin 3-glycosides are characteristic.

Malvidin gives a slightly bluer violet in the amyl alcohol-sodium acetate test than cyanidin and ferric chloride does not change it. Pure malvidin is, however, of rare occurrence. The oxidation test leaves malvidin largely unchanged and it is not extracted by the cyanidin reagent, but completely by the delphinidin reagent.

Petunidin gives a violet-blue in the amyl alcohol-sodium acetate test and pure blue after the addition of ferric chloride. It is destroyed in the oxidation test, not extracted by the cyanidin reagent and has a lower distribution than cyanidin in the delphinidin reagent. It is best recognised by successive extractions of a solution with small portions of the delphinidin reagent.

Delphinidin gives a blue solution in amyl alcohol on the addition of sodium acetate; it is destroyed in the oxidation test, not extracted by the cyanidin reagent or by the delphinidin reagent.

Hirsutidin is rare and if it occurs in any of the solutions tested it would be included under malvidin. It gives reactions similar to those of malvidin.

Colour reactions of the anthocyanins.

Pelargonin (and possibly other pelargonidin 3 : 5-dimonosides) is easily recognised. It gives a violet coloration with aqueous sodium carbonate and this becomes greenish-blue on the addition of acetone. Decisive confirmation is obtained by addition of $\frac{1}{4}$ volume of concentrated hydrochloric acid to the solution and boiling for $\frac{1}{2}$–1 minute; then on extracting with amyl alcohol a green fluorescence due to pelargonenin will be observed.

Pelargonidin 3-glycosides, for example, callistephin, give a violet-red coloration with sodium carbonate and this is rather stable towards sodium hydroxide. No other anthocyanin-type gives a similar reaction.

Peonin (3 : 5-type) gives a blue coloration with sodium carbonate. Peonidin 3-glycosides do not occur in the sequel, but the sodium carbonate reaction is a rich violet unchanged by sodium hydroxide.

Cyanin gives a rich pure blue coloration with sodium carbonate, unstable to sodium hydroxide, whereas mecocyanin (chrysanthemin) gives a blue-violet with sodium carbonate changing to pure blue with sodium hydroxide.

Malvin (3 : 5-type) gives a bright greenish-blue with sodium carbonate, whilst oenin (malvidin-3-glucoside) gives a blue-violet unchanged by sodium hydroxide.

The delphinidin glycosides have not been distinguished with the aid of their colour reactions in the absence of synthetic standards.

Very frequently the anthocyanin had to be purified before satisfactory reactions could be obtained.

Solutions of diglycosides could be exhaustively extracted with amyl alcohol and occasionally this was sufficient. Otherwise the pigment was taken up in amyl alcohol-acetophenone (2 : 1) in the presence of picric acid. This reagent was introduced by Willstätter and Schudel [1918]. The separated, filtered organic layer was agitated with 1 % hydrochloric acid and diluted with ether. Picric acid was then carefully removed from the aqueous solution by further extraction with ether. Monoglycosides were similarly purified by extraction with cyclohexanone and picric acid or with ethyl acetate and picric acid; the extracts were precipitated with light petroleum and the anthocyanins taken up in 1 % hydrochloric acid. The solution was usually extracted with benzene, then with cyclohexanone and again with benzene. Occasionally the process was repeated.

Summary of results.

Achillea Kelwayi. Complex diglycoside.

Aconitum napellus. Delphinidin diglycoside; contains much anthoxanthin.

Aesculus hippocastanum. The pink flowers contained a cyanidin 3-glycoside which required exhaustive purification. The distribution was intermediate suggesting a rhamnoglucoside or the like.

Althaea rosea. Various hollyhocks were examined and the results suggested mixtures of malvidin, petunidin and delphinidin mono- and di-glycosides. Delphinidin and malvidin were present beyond doubt and the occurrence of petunidin was inferred from the behaviour of the anthocyanidin on successive extraction with the delphinidin reagent.

Anchusa italica (Dropmore var.). The mauve buds and blue flowers gave the same results. The anthocyanin is essentially a delphinidin diglycoside, but the behaviour of the anthocyanidin with the delphinidin reagent indicated some methylation of the pyrogallol nucleus.

Anemone coronaria (St Brigid var.). Red flowers contained a pelargonidin 3-bioside and violet flowers contained cyanidin 3 : 5-dimonoside, possibly mixed with a malvidin derivative. The anthers of both varieties contain what appears to be the same anthocyanin characterised by a high distribution number. Further examination of the anemone is projected; this flower was studied before our system had been adequately developed.

Armeria maritima. Malvidin 3 : 5-dimonoside.

Arum maculatum. Anthocyanin absent.

Astilbe gloria purpurea. The reactions were much obscured by other substances but the anthocyanin proved to be a cyanidin 3-glycoside. The distribution was intermediate between that of mecocyanin and that of chrysanthemin.

Aubrietia deltoides. The purple flowers of the variety "Dr Mules" were found to contain a delphinidin diglycoside, whilst the rose-red Leichtlini was found to contain a cyanidin 3:5-dimonoside.

Begonia semperflorens. A fibrous-rooted bedder with crimson flowers gave a solution containing a cyanidin 3-bioside; the leaves of another variety with small pink flowers contained cyanidin 3-bioside.

Tuberous-rooted begonias have also been examined; the scarlet-flowered varieties contain pelargonidin 3-bioside and the crimson kinds, cyanidin 3-bioside.

Bellis (Rob Roy). A co-pigment makes the solution much too blue-red, but the anthocyanin is a cyanidin 3-bioside.

Berberis aquifolium. The deep purple fruits contain 3-monoglycosides of malvidin, delphinidin and probably also, petunidin. The anthocyanin gives a violet coloration in aqueous sodium carbonate and this becomes green on the addition of sodium hydroxide. Cyanidin is absent, but the reactions cited are those of cyanidin 3-glycosides, and it is evident that these changes may be observed in the delphinidin series also.

Buddleia amplissima. Delphinidin diglycoside.

Calceolaria. A magenta garden variety contained cyanidin 3-bioside and this was the main anthocyanin of a brown-red kind; in this case the cyanidin was partly replaced by pelargonidin.

Callistephus hortensis. A blue-violet Aster of the "Victoria" class was found to contain a delphinidin diglycoside. The characteristic pigments of other varieties are monoglycosidic. According to Fleming [1929] no true blue aster exists.

Campanula glomerata. Purplish-blue flowers contained a delphinidin diglycoside mixed with a small proportion of a reddening pigment. The variety "Telham Beauty" has blue-violet flowers and the anthocyanin was a delphinidin diglycoside.

Pink flowers of *Campanula Medium* were unexpectedly found to contain a pelargonidin 3-bioside; this anthocyanin is usually associated with a scarlet-coloured flower, the rose-pinks based on pelargonidin owing their colour to pelargonin or similar anthocyanin.

Carduus lanceolata. Cyanidin 3:5-dimonoside.

Centaurea nigra. The distribution number was zero, otherwise all tests showed that the anthocyanin was a cyanidin 3:5-dimonoside.

Chaerophyllum anthriscus. The purple leaves gave an extract containing a cyanidin 3:5-dimonoside.

Charies heterophylla. Delphinidin diglycoside.

Chrysanthemum coronarium. Pyrethrums such as "Countess Poulett," "Sutton's Blue Red," and "Mrs James Leake" were all found to contain a cyanidin 3-glycoside. There was a powerful co-pigment effect.

Chrysanthemum tricolor gave solutions having exactly the same properties.

Cichorium entybus. Delphinidin diglycoside.

Clarkia elegans. A coral pink variety yielded pelargonidin 3:5-dimonoside and a violet-flowered variety gave a very clean solution of pure malvidin 3:5-dimonoside. In this case the malvidin was free from any trace of a substance giving a positive ferric reaction.

Clematis Jackmani. The anthocyanin is a delphinidin diglycoside mixed with a relatively small proportion of methylated delphinidin derivatives.

Coleus Blumei. The leaves of several varieties were examined and all found to contain a complex diglycoside of cyanidin. The acid solution was very blue-red and developed a fine blue colour on the addition of sodium acetate.

The distribution was monoglucosidic, or even higher, and after purification the anthocyanin gave a reddish-violet with sodium acetate. The diglycoside resulting from the hydrolysis of the anthocyanin (cold sodium hydroxide in a hydrogen atmosphere) was purified and its distribution between butyl alcohol and 1 % hydrochloric acid was identical with that of cyanin. The whole of the reactions of the diglycoside supported the view that it is actually cyanin. So that coleusin is an acyl derivative of cyanin and the nature of the acid is being investigated.

Convolvulus sepium. Rose-heliotrope flowers contain a cyanidin 3-monoglycoside.

Crataegus oxyacantha fl-pleno rosea. The flowers are coloured by a cyanidin 3-bioside and the skins of the red berries contain a cyanidin 3-glycoside.

Dactylis glomerata L. The bluish-mauve flowers were separated and a cyanidin 3-bioside was diagnosed. The distribution is, however, intermediate between monoglucosidic and diglucosidic.

Delphinium. A number of the garden perennial varieties have been examined, for example, "Prince of Wales," "True Blue," "W. T. Ware," "Corry Ware," "Mrs Townley Parker" and *D. formosum*; also annuals derived from *D. consolida.* All gave evidence that delphinidin is the main anthocyanidin, but it is mixed in most cases with a small proportion of a methylated derivative.

The anthocyanins all exhibited the stability in aqueous sodium carbonate solution, which, Willstätter and Mieg [1915] showed to be characteristic of delphinin.

Dianthus barbatus. The bluish-red flowers of the type gave a blue-red acid extract and after exhaustive purification of the anthocyanin it was characterised as cyanidin 3-monoglycoside. The garden varieties (Sweet William) also furnished 3-monoglycosides; a crimson and also a black-crimson (*nigricans*) variety furnished a solution which could not be distinguished in distribution number tests from one of chrysanthemin chloride. A scarlet variety, however, was found to owe its colour to a pelargonidin 3-monoglycoside, probably callistephin and following that anthocyanin exactly in distribution number tests. These apparent monoglycosides were found to be such in fact, and do not owe their high distribution number to complex diglucosidic constitution.

Dianthus caryophyllus. We are greatly indebted to Mr E. M. Morgans for supplies of a number of varieties of carnations.

"Nigger" and "Topsy" (deep brown-red) owe their colour to a cyanidin 3-monoglycoside, the distribution number of which, after purification, was identical with that of chrysanthemin under the same conditions. "Brilliant," "Aviator" and "Spectrum" (scarlet) are similarly coloured by a pelargonidin 3-monoglycoside tallying with callistephin. "Mary Allwood" (salmon-red) also contains the pelargonidin 3-glycoside. But, surprisingly, "Improved Ward" (bluish-pink) contains a pelargonidin 3 : 5-dimonoside agreeing in distribution number (*n*-butyl alcohol) with pelargonin.

Dierama pulcherrima. Mauve-rose flowers contained a malvidin 3 : 5-dimonoside.

Digitalis purpurea. The anthocyanin is a cyanidin 3 : 5-dimonoside.

Dodecatheon integrifolia. Malvidin 3 : 5-dimonoside. The solutions contained much anthoxanthin.

Epilobium angustifolium and *E. hirsutum.* The willow herbs contain a malvidin 3 : 5-dimonoside and apart from the presence of a co-pigment these available flowers provide good standards for malvin-type reactions.

Erigeron speciosus. A very strong co-pigment effect was noted; the anthocyanin is a delphinidin diglycoside.

Fagus sylvatica. Leaves of the copper beech give blue-red extracts and were found to contain a complex cyanidin 3-bioside. The distribution number of this anthocyanin closely approaches that of chrysanthemin for amyl alcohol, ethyl acetate in presence of picric acid and butyl alcohol, all with 1 % hydrochloric acid. A considerable divergence was, however, noted when the organic solvent was *n*-butyl alcohol mixed with $\frac{1}{2}$ vol. of benzene. The beech anthocyanin had the higher distribution number. The complex nature of the pigment was proved in the usual manner and an attempt to identify the acid concerned will be made in due course. This is the first example of a complex diglycoside derived from a 3-bioside anthocyanin.

Fragaria vesca. The colour of strawberries is due to a pelargonidin 3-monoside.

Fuchsia. The purple petals of the inner corollae of several varieties (for example, "Rose of Castile") gave identical results which have already been described. The anthocyanin is mainly a malvidin 3 : 5-dimonoside but the alkali-colour reactions were not quite standard, being too red even after purification of the pigment through the picrate. The anthocyanidins concerned could be pelargonidin and peonidin only, since the ferric reaction was quite negative.

A clue was obtained by studying the bluish-red outer petals which were found to contain a peonidin 3 : 5-dimonoside mainly, mixed with a blueing component. We then found that the reactions could be perfectly matched by adding a little peonin to malvin for the inner petals or a little malvin to peonin for the outer petals. The use of pelargonidin 3-bioside from *Papaver orientale* gave a very poor match and pelargonidin 3 : 5-dimonoside cannot be present, since no pelargonenin-type fluorescence could be observed. The absence of pelargonidin from the fuchsia

anthocyanidins was confirmed by adding saturated aqueous picric acid (1 vol.) to a solution in 0·5 % hydrochloric acid (1 vol.) and shaking with a mixture (1 vol.) of *iso*propyl ether with 3 vols. of *iso*amyl ether. The upper layer was not coloured by the anthocyanidin, but after adding a few drops of a dilute solution of pelargonidin chloride and again shaking, the organic layer was coloured by anthocyanidin.

Carrying out the same experiment but using a mixture of equal volumes of *iso*propyl ether and *iso*amyl ether, the presence of peonidin was indicated in the anthocyanidins from both types of petals. This mixture distinguishes peonidin from malvidin in dilute solutions; in the former case the upper layer assumes a bluish salmon-red colour but malvidin is not extracted, although the picrate may be precipitated. The variety "Corallie" with long, tubular, salmon-red flowers was found to contain a pelargonidin 3 : 5-dimonoside.

Galega officinalis. The violet flowers contain a delphinidin diglycoside.

Geranium pratensi. Malvidin 3 : 5-dimonoside.

Geranium Robertianus. The leaves and stem furnished an extract of a cyanidin 3-bioside, whilst the bluish-pink flowers contained a malvidin 3 : 5-dimonoside.

Geum chiloënse. The varieties "Mrs Bradshaw" and "Orange Queen" gave the same results. The anthocyanin is a cyanidin 3-monoside but there are indications of the presence of a small proportion of a pelargonidin derivative.

Gladiolus gandavensis. This group exemplifies well our thesis that colour variations are usually due to the appearance of different anthocyanins. The salmon-pink varieties all contain a pelargonidin 3 : 5-dimonoside. In some cases, for example, "Flaming Sword," the alkali-colour reactions suggest admixture with a small proportion of a peonidin or cyanidin derivative. In the scarlet sorts, for example, "Scarletta," we find a pelargonidin 3-bioside. The distribution number is lower than that of callistephin but is higher than that of the pelargonidin bioside in the gloxinia and scarlet cineraria.

A crimson-flowered variety contained an anthocyanin, the distribution number of which was zero; it gave all the reactions of a cyanidin 3 : 5-dimonoside. The occurrence of a malvidin 3 : 5-dimonoside was noted in a purple-red variety (Jacob von Bengeren); this contained a trace of an anthocyanin giving a positive ferric reaction.

G. primulinus, a red-violet variety, was also found to contain a malvidin 3 : 5-dimonoside.

Gloriosa vivescens Rothschildiana. Specimens kindly supplied by Lord Rothschild were found to contain cyanin, which in this case was isolated and identified. There is also a bright yellow anthoxanthin.

Gloxinia. "Reading Scarlet" and "Beacon" contain a pelargonidin 3-bioside, whilst "King George" is coloured by the same pigment together with a small proportion of an anthocyanin giving more blue-red acid solutions. "Purple Sultan" and "Violet" yield diglycosidic anthocyanins based on malvidin but containing some cyanidin, petunidin or delphinidin derivative. The alkali-colour reaction was not precisely of malvin-type and it may be that this is due to the rare malvidin 3-bioside constitution or more probably to lack of homogeneity.

Hyacinthus orientalis. "King of the Blues" contains a delphinidin diglycoside and "Queen of the Pinks" contains a pelargonidin 3 : 5-dimonoside. Neither anthocyanin is homogeneous, the evidence being in favour of the presence of a cyanidin derivative in both violet-blue and pink flowers.

Hydrangea hortensis. Considerable interest attaches to a study of the pigments of these flowers on account of the popular idea that iron in the soil helps in the development of the blue colour. Unfortunately the blue kinds do not produce their pigment lavishly, but we have been able to form an opinion respecting the relation between the respective colouring matters.

A red-flowered variety, "Parzival," gave a bluish-red solution containing a co-pigment and the anthocyanin was a delphinidin derivative. As the distribution number seemed rather high for a delphinidin diglycoside, tests for complex diglycosidic character were made and these gave a positive result, the recovered diglycoside having a distribution number approximating to zero. The anthocyanin from a blue-flowered variety, "Maréchal Foch," had a much lower distribution number than that from the red flowers. It proved to be a delphinidin diglycoside. It is noteworthy that the colour from the red flowers is sensitive to co-pigments, whereas that from the blue flowers is much less so.

Iberis umbellata. The purple flowers contain a cyanidin 3 : 5-dimonoside mixed with a relatively small proportion of a malvidin derivative.

Ilex aquifolium. The skins of holly berries contain a pigment which tallies best with a pelargonidin 3-bioside; a further examination is necessary.

Iris Kaempferii. A bluish-purple variety of the Japanese Iris contained a malvidin 3 : 5-dimonoside mixed, probably, with a petunidin derivative. Several unnamed blue and violet varieties of the German Iris furnished solutions of delphinidin diglycoside much blued by co-pigments and the variety "Imperator" gave identical results.

Ixia crateroides. The cerise-coloured flowers contain a cyanidin 3 : 5-dimonoside and this is probably cyanin, since a comparison of the purified pigment with cyanin in respect of its distribution number (*n*-butyl alcohol—1 % hydrochloric acid) showed that these were identical.

Lathyrus. L. grandiflorus, crimson flowers, develops a mixture of diglycosidic anthocyanins; the anthocyanidins consist of malvidin (less than 40 %) and delphinidin, whilst the presence of petunidin may be inferred from the behaviour of the anthocyanidin solution on extraction with successive small volumes of the delphinidin reagent. *L. latifolius* is coloured by nearly homogeneous malvidin 3 : 5-dimonoside. *L. odoratus* provides one of the most interesting groups and we propose to study it in some detail; the solutions are clean and the petals are easily extracted.

For named varieties of Sweet Peas as also for Gloxinias, Cinerarias, Salpiglossis, *etc.*, we are very greatly indebted to Messrs Sutton and Sons of Reading. "2 LO," scarlet, and "Glorious," pink, gave solutions of a pelargonidin 3-bioside. "Crusader," bluish-pink, contains, however, pelargonidin 3 : 5-dimonoside. "Captain Blood," blood-red, gives solutions containing more anthoxanthin than usual in the group. Its chief anthocyanin is cyanidin 3-bioside, but this is mixed with a small proportion of pelargonidin 3-bioside. Cyanin and pelargonin types of anthocyanins were not present. "Damask Rose," bluish-red, contains a mixture of pelargonidin and cyanidin 3 : 5-dimonosides. The same is true of "Sybil Henshaw," bluish-red, but in this case the proportion of cyanidin derivative is higher than in "Damask Rose." A match for all the reactions was obtained using 70 % of cyanin and 30 % of pelargonin. No match could be made using mecocyanin. Doubtless a variety yielding good cyanin-type reactions free from pelargonin exists but, even although we have not yet encountered it, we consider the proof of the occurrence of cyanidin 3 : 5-dimonoside is satisfactory.

It thus appears that the Sweet Peas contain both types of pelargonidin and cyanidin diglycosides and these are by no means all. "Bacchus," wine-red, possesses colouring matters based on methylated delphinidins, delphinidin itself appears to be absent. Cyanidin is also absent and we consider that the chief anthocyanins are malvidin and petunidin 3 : 5-dimonosides. The evidence for malvidin is the result of the oxidation test and the behaviour towards the delphinidin reagent; petunidin is indicated by the latter and by the positive ferric reactions, cyanidin and delphinidin being ruled out.

"Chieftain," light red-purple, contains chiefly malvidin 3 : 5-dimonoside and the same is true of "Royal Purple," red-purple; these two give weak ferric reactions as purified anthocyanin or as anthocyanidin. "The Flag Lieutenant," purple, and "Black Diamond," deep red-purple, furnish anthocyanins giving a strong ferric reaction; malvidin and delphinidin were proved to be present and cyanidin was absent. The existence of petunidin in the mixture is strongly suspected but we cannot put this forward with confidence. The anthocyanins are all diglycosidic and the alkali-colour reactions are of the malvin type.

"Blue Bird," violet-blue, contains a delphinidin diglycoside as the major anthocyanin.

Lavandula vera and *L. nana* both contain a delphinidin diglycoside.

Lavatera trimestris rosea splendens. This anthocyanin will be further examined; it appears to be based on petunidin. The oxidation test on the anthocyanidin shows that cyanidin and malvidin are absent, yet the ferric reaction is positive and the distribution with the delphinidin reagent is approximately correct for petunidin. Peonidin and pelargonidin are absent. Moreover, the reactions of the anthocyanin, which has a high distribution number, agree with the suggestion that it is petunidin 3-monoside. We have not excluded the complex diglycoside possibility in this case.

Lilium Martagon. The reddish-purple flowers and also the stems yielded a blue-red solution containing a co-pigment. The anthocyanin is a cyanidin 3 : 5-dimonoside.

Lilium umbellatum (Dahurian Lily) has orange flowers but the anthocyanin is a cyanidin

3-bioside. It required exhaustive purification on account of the presence of much anthoxanthin and other substances.

Linum. A crimson-scarlet-flowered annual variety (*grandiflorum* var. *rubrum*) was found to contain a delphinidin diglycoside. In this case we may be justified in stating that the anthocyanin is a 3-bioside, since the coloration in sodium carbonate is royal blue and not greenish-blue as usual. There seems to be little co-pigment in this case and for these reasons we propose to attempt the isolation of this anthocyanin. It is remarkable that a flower so far removed from blue should contain an anthocyanin based on unmethylated delphinidin. Nevertheless, a little cyanidin appears to be present.

Linum narbonense, blue, contains a delphinidin diglycoside and, by contrast with the preceding, this is a 3 : 5-dimonoside and gives a greenish-blue coloration in sodium carbonate; also the colour in sodium acetate is violet-blue, whereas in the case of the annual scarlet flax it was violet.

Lupinus polyphyllus. The varieties "Pink Perfection," "H. Marshall," reddish-violet, and "Downers Delight," dull brownish salmon-red, contained a cyanidin 3-bioside. "King of the Blues," reddish-blue, contains a delphinidin complex diglycoside; "Sutton's Saxe Blue," "Sutton's Ruby" and "Amethyst" from Brookside Nurseries, Oxford, give quite similar results. "Victoria," bluish-dark purple, is also a complex diglycoside and the results suggest that it is a petunidin derivative. This conclusion is provisional.

Lychnis chalcedonica. The anthocyanin is a pelargonidin 3-glycoside with a distribution number intermediate between callistephin and pelargonidin 3-bioside.

Lythrum salicaria. "Brightness" contains a malvidin 3 : 5-dimonoside and a trace of an anthocyanin having a positive ferric reaction.

Matthiola incana. Some single and double ten-week stocks have been examined and all the anthocyanins therein have high distribution numbers and were found to be complex diglycosides. A magenta single-flowered variety furnished an acyl derivative of a pelargonidin 3 : 5-dimonoside; this had a distribution number > 50. A violet-coloured variety contained an acylated cyanidin 3 : 5-dimonoside. A deep purple double-flowered kind furnished an anthocyanin with a distribution number nearer to that characteristic of a diglycoside but still too high. On hydrolysis this yielded an anthocyanin not to be distinguished in distribution and other tests from cyanin.

It is noteworthy that the solutions of all these complex diglycosides of *Matthiola* are much too blue-red and that heating the solutions has a relatively small effect. We consider that in these cases the attached phenolic acid residue exerts an intramolecular effect similar to the intermolecular effect of the co-pigments. Coleusin is under all circumstances much bluer red in solution than cyanin.

Myosotis dissitiflora. "Blue King" contains a delphinidin diglycoside.

Nepeta mussini contains a complex diglycoside of delphinidin; the only difficulty was that the sodium acetate-amyl alcohol test on the anthocyanidin gave rather too red a shade. Other tests, however, confirmed the diagnosis of delphinidin.

Nepeta okrani also contains a complex diglycoside of delphinidin, but in this case the evidence of contamination is still more definite; also the distribution number of the original anthocyanin is higher than in the case of that from *N. mussini*.

Nemophila insignis. Delphinidin diglycoside.

Nigella damascena. Malvidin 3 : 5-dimonoside.

Oenothera amoena (syn. *Godetia rubicunda*). A bluish-red variety gave diglycosidic anthocyanins, certainly 3 : 5-dimonosides, but apparently not homogeneous and derived from malvidin and cyanidin (or petunidin).

Origanum vulgare. The anthocyanidin is very definitely malvidin and it is found unmixed with other anthocyanidins; further the anthocyanin is diglycosidic. The characteristic malvin-type alkali-colour reaction could not be obtained and the colour with sodium carbonate was violet, but this is possibly due to impurities not removed in the cyclohexanone-picric acid process.

Papaver orientale bracteatum. The orange-scarlet flowers, after removing all the dark central parts, gave a solution of a pure pelargonidin 3-glycoside. The colour reactions matched those of callistephin. The distribution number, however, was zero and the distribution number with solvents other than amyl alcohol was also abnormally low. Perhaps this anthocyanin is a trioside. The epidermis of the dark portions gave a solution of cyanidin 3-bioside, doubtless mecocyanin

and the anthers also contained the cyanidin derivative. A pink variety, "Queen Alexandra," also contained a pelargonidin 3-bioside and the explanation of the colour change is probably to be found in the presence of a co-pigment.

Many other varieties of poppies have been examined and in general the pink and scarlet kinds (also the orange, *Papaver nudicaule*) contain pelargonidin derivatives, always 3-glycosides, whilst the deep red and blue-red varieties contain a cyanidin 3-bioside (mecocyanin). The darker portions are never free from mecocyanin and this may, or may not extend to other parts of the petals. A common form of the field poppy (*P. rhoeas*) has scarlet flowers, the petals of which (excluding the splashes) yield solutions containing both pelargonidin and cyanidin derivatives. Pink Shirley poppies give blue-red acid solutions, but these are co-pigmented and purification of the anthocyanin through the picrate yields normal pelargonin-like solutions.

Mecocyanin [Willstätter and Weil, 1916] may, as usually prepared, be accompanied by an impurity; its alkali-colour reaction can be matched by a solution made by adding about 12 % of cyanin to chrysanthemin. On the other hand, this tendency to give slightly bluer reactions than chrysanthemin may be an inherent property of the substance.

Pentstemon. "Southgate Gem" and *isophyllis* gave scarlet-red solutions having similar reactions; a strong co-pigment was present. The anthocyanin is a pelargonidin 3 : 5-dimonoside, the distribution number of which is higher than that of pelargonin and lower than the complex diglycosides of this group.

Petunia. In view of our section on the existence of petunidin, it is interesting to note that permanganate-coloured petunias furnished an anthocyanin of the methylated delphinidin type, but this gave, on hydrolysis, an anthocyanidin consisting of malvidin to the extent of at least 57 %. The remainder was probably petunidin, but this could not be proved in the presence of so much malvidin. Willstätter and Burdick [1916] used a special blue petunia and their process of isolation may have had much to do with the successful removal of anthocyanins based on malvidin and delphinidin.

Phlox. A few varieties of herbaceous Phlox have been examined; they are remarkable for the efficiency of their co-pigments.

"Jules Sandean," pink, contains a pelargonidin 3 : 5-dimonoside and a trace of a cyanidin derivative; "Leo Schlageter," crimson-scarlet, contains the same anthocyanin with a little more cyanidin derivative. On heating, the solutions in 1 % hydrochloric acid became much more orange-red and the blue-red colour returned on cooling. This property was not destroyed by heating with sodium hydroxide until bright yellow, boiling the solution for a few seconds, and then reproducing the anthocyanin. The solution was still blue-red and the alternation of colour on heating and cooling could still be brought about. The co-pigment is therefore not an ester. A blue-violet "Iris Phlox" was found to owe its colour to a malvidin 3 : 5-dimonoside.

Potentilla Willmottiae. Cyanidin 3-bioside; the distribution number suggests the possibility of rhamnoglucoside. Other potentillas having a like colour or deep brown-red colour, gave similar results.

Poterium obtusum. The blue-red flowers contain a cyanidin 3 : 5-dimonoside.

Primula Juliana contains a malvidin 3 : 5-dimonoside and *P. sinensis*, "Reading Ruby," also contains this type of anthocyanin.

Prunus Avium. The skins of small black cherries contain cyanidin 3-monoside; the distribution number is higher than that of keracyanin [Willstätter and Zollinger, 1916].

Prunus communis. The skins of a deep purple plum were found to contain a cyanidin 3-monoside. The variety was "Rivers Early."

Prunus Pissardii. The leaves contain a cyanidin 3-monoside; there was no complex diglycoside.

Pyrus malus. The skins of the apples "Wine Sap" and "Jonathan" were found to contain a cyanidin 3-monoside. The flowers of "Niedzwetzkyana" also yield a cyanidin 3-monoside.

Rheum rhaponticum (Sutton's rhubarb). The colouring matter in the skin of the stem is a cyanidin 3-bioside.

Rhododendron indicum. A salmon-red azalea gave an extract singularly free from anthoxanthins and the colouring matter was a cyanidin 3-monoside. It was almost certainly chrysanthemin, since its distribution numbers under four sets of conditions were identical with those of chrysanthemin. The solvents employed were amyl alcohol, butyl alcohol with 20 vols. % of benzene, ethyl

acetate in the presence of picric acid in small and also in large concentration; all were put in competition with an equal volume of 1 % hydrochloric acid.

Ribes. Willstätter and Bolton [1916] found that the berries of *R. rubrum* L. contained a cyanidin diglucoside. This is a cyanidin 3-bioside and it is accompanied by a pelargonidin or peonidin derivative. A curious feature was the difficulty experienced in getting a positive ferric reaction with the original solution.

The skins of large red gooseberries (*Ribes grossularia*) contain a cyanidin 3-monoside and the black currant (*Ribes nigrum*) contains a cyanidin 3-bioside. In the latter case there is much co-pigment in the original solution and normally coloured solutions result from purification of the anthocyanin through the picrate. Cyanidin was the only anthocyanidin found, but it should be noted that a small percentage of delphinidin could not in any case be detected and the related anthocyanin would probably be eliminated in the purification process.

Rosa. Young red leaves of the rose contain a cyanidin 3 : 5-dimonoside, doubtless cyanin.

Rubus idaeus. Willstätter and Bolton [1916] diagnosed a cyanidin glucoside in the raspberry; it is a cyanidin 3-bioside. Loganberries also contain a cyanidin 3-bioside and in both cases the reactions represent a close approach to those of pure mecocyanin.

Rubus odoratus. An interesting anthocyanin occurs in the flowers. It has a high distribution number and on hydrolysis yields a cyanidin 3 : 5-dimonoside indistinguishable in its reactions and distribution between *n*-butyl alcohol and 1 % hydrochloric acid from cyanin. This complex cyanidin diglycoside is probably not identical with that found in *Coleus Blumei*; its solutions though blue-red are not markedly so as in the latter case, and the reactions with sodium acetate are strikingly dissimilar. The anthocyanin under consideration gives a violet-red and that from *Coleus* leaves almost a pure blue coloration. The difference must reside in the nature or mode of attachment of the associated acid and further investigation is projected.

Rumex acetosa L. Cyanidin 3-bioside.

Salpiglossis sinuata. Sutton's "Large Blue-flowered" and "Light Blue and Gold" contain a delphinidin diglycoside. The reactions of the solutions from "Velvet Red" and "Black Knight" agree best with the view that they contain mainly petunidin diglycosides.

Salvia splendens. The variety "Grahamei" has crimson flowers and as the more orange-scarlet types were employed by Willstätter and Bolton [1916] it is of interest to note that this variety, also, contains a complex pelargonidin 3 : 5-dimonoside.

Salvia patens. Delphinidin diglycoside, possibly complex.

Salvia pratensi. The anthocyanin appears to be not homogeneous; it is mainly a complex malvidin 3 : 5-dimonoside, but there is also a substance present which gives a ferric reaction.

Salvia virgata nemorosa. The interesting anthocyanin is a complex malvidin 3 : 5-dimonoside; it is very blue-red in acid solution and is relatively difficult to hydrolyse by means of acids, in which respect it resembles the anthocyanin of *Coleus*. The diglycoside obtained after alkaline hydrolysis and recovery by means of hydrochloric acid is probably malvin. The acid of the complex has been isolated and is being examined.

Saponaria. A scarlet-crimson annual variety gave a solution which contained a cyanidin 3 : 5-dimonoside and very satisfactory reactions were obtained with the original unpurified material.

Saxifraga sanguinea superba. The solution was blue-red and the anthocyanin had a high distribution number. It yielded cyanidin on hydrolysis but did not tally with any of the known types.

The reaction with sodium acetate is blue-violet even after purification through the picrate, and the violet colour with sodium carbonate becomes blue on the addition of sodium hydroxide, but not so readily as in the usual chrysanthemin-type cases. There is some novel feature in connection with this anthocyanin and next season a closer investigation is proposed.

Scabiosa arvensis. Delphinidin diglycoside.

Scrophularia nodosa. This figwort contains a cyanidin 3-bioside and much anthoxanthin.

Solanum dulcamara. The purple flowers contain a delphinidin diglycoside which gives a deep blue-violet with sodium acetate and so is probably a 3 : 5-dimonoside.

Senecio (*Cineraria stellata*). Deep blue hybrids contain a delphinidin diglycoside (distribution number zero), and the aqueous acid extracts become pure blue on the addition of sodium acetate. Similar results were obtained with named varieties, for example, "Forget-me-not Blue," "Royal

Blue," "Blue Gem," "Blue with White Zone" and "Duchess Star." On the other hand, the rich purple flowers give an extract that develops a corresponding blue-violet on the addition of sodium acetate. The distribution number is still zero and the anthocyanidin is delphinidin. Possibly the change is in the attachment of carbohydrate residues. Cineraria "The King" and "Brilliant Ruby" contain the same anthocyanin which has a low distribution number (but not zero), and appears to be a malvidin derivative mixed with a substance giving a positive ferric reaction. The reactions do not, however, tally with those of known types and this, together with the blue cineraria, will be further studied. Cineraria "Pink Pearl" and the large scarlet-flowered variety contain a pelargonidin 3-bioside unmixed with other anthocyanins.

Silene angelica. The flowers of this catchfly contain a malvidin 3 : 5-dimonoside mixed with a trace of a cyanidin derivative, and the stems contain a cyanidin 3-bioside with a trace of a malvidin derivative. Attention is directed to the fact that this distribution of a malvin-type and meconcyanin-type of anthocyanin has also been observed in the *Geranium Robertianus* (Herb Robert).

Sistus purpurea and *Sistus* "Sunset" appear to contain a cyanidin 3-bioside, but the change of colour from violet to blue on adding sodium hydroxide to a solution containing sodium carbonate was obscured by impurities not removed by the ordinary processes.

Stachys sylvatica. Cyanidin 3-monoside.

Statice sinuata. The violet-blue flowers yielded nothing to 1 % hydrochloric acid and had to be ground with the solvent in a mortar. (Note: this is not usually a good practice unless the solutions are centrifuged, when it is time-saving.) The very blue-red extract was found to contain a delphinidin diglycoside unmixed with other anthocyanins.

Tricuspidaria dependens. The reactions of the anthocyanin from this crinodendron were much obscured but the anthocyanidin is cyanidin, the distribution number was very low and after exhaustive purification the cyanidin 3-bioside reactions were observed, although not in the usual unambiguous manner.

Trifolium. "Sutton's Early Red Clover" gives an extract containing a cyanidin 3-bioside.

Tritoma uvaria. The flowers do not contain anthocyanin.

Tropaeolum majus L. Willstätter and Bolton [1916] stated that the flowers contain a cyanidin derivative; this is true of the deep brown-red variety and the anthocyanin is a cyanidin 3-bioside. The common orange-scarlet variety contains a pelargonidin 3-bioside and the anthocyanins are thus like those of the poppy.

Tulipa Gesneriana L. This is a very complex group characterised by mixtures of anthocyanins. Willstätter and Bolton [1916] found that the anthocyanin of a dark red garden tulip was cyanin, occurring both in the petals and the anthers. We are quite prepared to accept this finding (especially in view of our experiences with the sweet peas and gladiolus), but have not ourselves encountered a similar variety. We are much indebted to Sir Daniel Hall for supplies of varieties of tulips and the present is an interim report; the examination of the tulips is much more difficult than that of most other groups.

The varieties "Orange Perfection," "D," "City of Haarlem" and "Inglescombe Scarlet" constitute a group in which the main anthocyanin is pelargonidin 3-bioside, but it is mixed with some cyanidin 3-bioside in all cases. A variety "Electra" is typical of a middle group in which the main anthocyanin is a cyanidin 3-bioside and the subsidiary pigment, present in substantial relative amount, is pelargonidin 3-bioside. Other members of this group are probably "Dorothy Ann," "Couleur Cardinal," "Proserpine," "Cecilia," "28" and "Mary Swan." "Venus," "Rhoda Backhaus" and "Eos" also contain cyanidin and pelargonidin 3-biosides, but in these cases we did not gain an idea as to which predominated. At the other end of the scale "William Copland" was found to contain a delphinidin diglycoside and similar varieties are "Daphne," "Lucy Lund," "Widor" and "916." The greater number of varieties are intermediate between the cyanidin 3-bioside group and the delphinidin diglycoside group. They are free from pelargonidin derivatives and comprise "Giant," "Isobel," "Velvet King," "Orange Girl," "Julietta," "Prancing Nigger," "47," "29," "Cecil Dolling," "Lavinia," "Philip de Commines," "Grey Dawn," "Perdita," "Zulu," "La Noire," "Sultan." All are diglycosidic and none is homogeneous. The anthocyanidins derived from "La Noire" and "Sultan" have been separated in some quantity and the result of their examination is to suggest that they consist of petunidin and delphinidin with a trace of malvidin, but work on this topic is still in progress. A summary of the results is that in the

tulips we find a group in which pelargonidin and cyanidin occur as 3-biosides and then apparently with a rather sharp separation a second group containing delphinidin derivatives.

The varieties "Grey Dawn" and "Perdita" were examined in "breeder" and in "broken" condition; in both cases the extracts from the former were bluer but no great differences could be detected in the anthocyanins.

Urtica dioica. The flowers contain a cyanidin 3 : 5-dimonoside.

Verbena, Crimson hybrid. A cyanidin 5-dimonoside was diagnosed, but this case presented some curious features in that hydrolysis was difficult and the solutions were much too blue-red.

Vicia cracca. Delphinidin diglycoside.

Viola gracilis and other violas contained a delphinidin diglycoside, doubtless violanin in many cases. The causes of colour variation in this family, apart from the presence of carotenoids and anthoxanthins, appear to be methylation of the delphinidin nucleus and the presence or absence of co-pigments. Thus "Pickerings Blue" gives a very blue-red acid extract and the redder-violet *Viola cornuta* yields a redder acid solution. But on heating the two solutions are identical in tone and the colour difference returns on cooling. The co-pigment present in "Pickering's Blue" can be removed and introduced into the solution from *Viola cornuta*; the situation is then reversed. Evidently the difference in colour in this case is purely a question of the co-pigment.

In other cases of very red-violet violas it was shown qualitatively by the micro-Zeisel method that the anthocyanidin is partly methylated. Cyanidin derivatives were not encountered in the violas.

Weigela (*Diervilla hybrida*, Eva Rathké). The crimson flowers contain a cyanidin 3-monoside.

Zapania nodiflora. Delphinidin diglycoside.

Some observations on co-pigments.

The following substances have little or no effect in low concentrations on the colour of dilute acid solutions of oenin chloride: alanine, asparagine, nicotinic acid, quinolinic acid, anthranilic acid, *m*-aminobenzoic acid, *p*-aminobenzoic acid, phthalic acid, benzilic acid, glucose, raffinose, maltose, starch, inulin, methylglycoside, rutin (possibly due to sparing solubility), euxanthic acid (same comment), helicin, pyridine, 2 : 4-dihydroxyquinoline, catechol, hesperidin.

The following substances have a slight blueing effect on oenin solutions. Tyrosine, 2-hydroxy-3-naphthoic acid, protocatechuic aldehyde, pyrogallol, orcinol, salicylic acid, *p*-hydroxybenzoic acid, arbutin, euxanthic acid, baptisin, 2-hydroxyanthraquinone, β-methylglucoside, β-naphthol glucoside, catechin. The following have a moderately powerful effect. *m*- and *p*-Hydroxybenzaldehyde, β-resorcylic acid, gentisic acid, protocatechuic acid, vanillin, phlorhidzin, aloin, quercitrin, 4-hydroxyxanthone glucoside. Aesculin, ethyl gallate and tannin have a strong effect and the most active compound tested was 2-hydroxyxanthone glucoside; it is far more active than the isomeride. 2-Hydroxyxanthone glucoside also exerts a strong deepening and blueing effect on the colour of anthocyanins derived from cyanidin and the order of efficiency is cyanin > chrysanthemin > mecocyanin. The action on pelargonin is to darken the solution but not to make it more blue-red. Quercitrin blues oenin but does not increase the tinctorial intensity. We are indebted to Dr A. Robertson for pure specimens of his synthetic glucosides.

The authors wish to thank numerous friends for providing material and for assistance in the identification of specimens. They are deeply indebted to

Geheimrath Prof. R. Willstätter and to Prof. P. Karrer for specimens of anthocyanins. They have been greatly assisted by the generous help of Messrs Sutton and Sons of Reading.

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CLXXXIII. AN ANTHOCYANIN OF *OXYCOCCUS MACROCARPUS* PERS.

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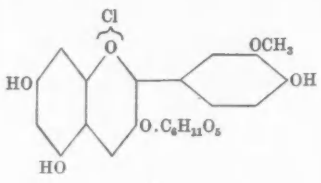
(*Received August 24th, 1931.*)

Having effected a synthesis of idaein chloride we wished to secure a supply of the natural pigment which Willstätter and Mallison [1915] isolated from the fruits of *Vaccinium vitis idaea* Linn. (this is the English cowberry, the true cranberry with smaller fruits being *V. oxycoccus*), but the latter not being in season we secured supplies of the much larger American cranberries in the hope that these might be a source of idaein.

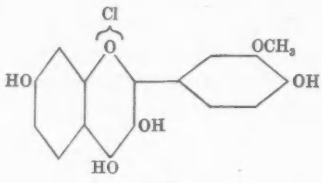
The first berries employed originated in Newfoundland and had a generally similar appearance to the second batch which came from the Cape Cod district.

The two were worked up separately, and the derived pigments were only combined when it was found that they had identical properties.

We have obtained the pure anthocyanin in small quantity only, but sufficient to establish its identity as a 3-*β*-*glucosidylpeonidin chloride* (I). On hydrolysis it yielded peonidin chloride (II), characterised by its analysis, habit of crystallisation and other properties, and by its colour reactions in buffered solutions.



(I)



(II)

The determination of the nature of the carbohydrate residue could not be made directly because of the paucity of material for investigation, but it happened that 3-*β*-glucosidylpeonidin chloride had been synthesised (Levy and Robinson) some time before the oxycoccicyanin was completely purified and direct comparison has shown that the two specimens are identical.

The most probable alternative to the glucosidic configuration is the galactosidic, and we therefore synthesised 3-*β*-galactosidylpeonidin chloride (paper submitted to the Chemical Society) and found that this anthocyanin closely resembled the glucoside. It differed, however, in its "distribution number" being less readily extracted by amyl alcohol from a solution in 0·5 % hydrochloric acid than is the glucosidic anthocyanin.

The new anthocyanin fills a gap in the series of monoglucosides, being a methoxycallistephin and a *des*methoxyoenin; we propose to designate it "oxycoccicyanin" following a precedent established by Nolan in naming "sambucicyanin." The alternatives are unsatisfactory, since "oxycoccin" suggests, perhaps, a derivative of carminic acid and "macrocarpin," referring simply to the size of the fruits, is not sufficiently specific.

Experimental.

(A) The cranberries from Newfoundland were rather smaller than those from Cape Cod; they were minced and submitted to great pressure in linen bags. We are indebted to Prof. J. C. Drummond for giving facilities and assistance at this stage. The skins were then extracted with acetic acid and the filtered extracts precipitated by the addition of ether. The syrupy material was again dissolved in acetic acid and precipitated by means of ether. The sticky red mass was then triturated with 2 % ethyl alcoholic hydrogen chloride and the solution filtered and precipitated by means of ether; this precipitate was dissolved in hot saturated aqueous picric acid and the solution kept in the ice-chest for several weeks. The crude picrate was extracted by means of boiling methyl alcohol and hydrogen chloride (to make a 1 % solution) introduced; the filtered solution was then mixed with an equal volume of ether. This precipitated impurities, including a cyanidin derivative (positive iron reaction); the filtrate from these was diluted with much ether and some light petroleum was also added. The intention had been to precipitate the chloride but, instead, the *picrate* crystallised on the sides of the vessels, which were kept for some weeks. The picrate could now be crystallised from hot ethyl alcoholic aqueous picric acid in red needles. After allowing ample time for complete separation the salt was collected, dissolved in hot methyl alcohol containing 2 % of hydrogen chloride and the chloride precipitated by means of ether. This gave a nearly pure amorphous specimen with a dark beetle-green lustre.

(B) The peelings of Cape Cod cranberries (60 lb.) were allowed to stand in 2 % methyl alcoholic hydrochloric acid for 2 to 3 weeks, using the counter flow method with the solvent. The solution was then filtered, the solvent being carefully pressed from the peelings, which were then washed with fresh solvent, the filtrate being added to the original solution. The colour was not entirely removed, but the skins were very light coloured after this treatment.

The alcoholic solution (*ca.* 11,000 cc.) was then divided into portions of 500 cc. and each portion was treated as follows: dry picric acid (10 g.) was added and the mixture was warmed to 40° and then poured into a large evaporating dish. Concentrated ammonium hydroxide (20 cc., the amount calculated almost to neutralise the acid present) was added and the mixture was thoroughly stirred and then allowed to stand in a draught chamber for 2 weeks; at the end of that time there was a crystalline residue covered with a sticky gum. The residues were collected, washed with saturated aqueous picric acid (1000 cc.), and quantities of ether, and dried (200 g.) (X).

This product was divided into 25 g. portions and each portion was boiled with absolute methyl alcohol to remove the pigment present. The combined extracts (*ca.* 1500 cc.) were filtered and dry ether (3 vols.) was added to the filtrate. A heavy precipitate formed on the addition of the ether, and this was filtered off and dried; very little anthocyanin was found in this material.

The filtrate was diluted with an equal volume of light petroleum (B.P. 40–60°) and the anthocyanin extracted by means of 1 % hydrochloric acid (500 cc.). The solution was then evaporated in a vacuum at the ordinary temperature and the picrate slowly separated. This was collected, dried and dissolved in the minimum of 3 % ethyl alcoholic hydrogen chloride; the solution was kept, filtered and the chloride precipitated by the addition of ether; the last process was repeated. The anthocyanin at this stage was compared with the product of process (A) from the Newfoundland berries and found to be entirely identical in alkali-colour reactions and in distribution number, using amyl alcohol with aqueous hydrochloric acid and ethyl acetate with aqueous picric acid. The specimens were therefore combined (see below).

The pigment was completely extracted from a typical berry with formic acid (1·5 cc.), the solution was filtered and diluted to 50 cc. with absolute methyl alcohol, and the amount of pigment determined colorimetrically by comparison with a solution of 2·0 mg. of idaein in methyl alcohol (50 cc.). The two solutions were approximately identical in colour. A pound of berries contains approximately 500 berries, *i.e.* 1·0 g. of pigment.

A determination of the relative amounts of anthocyanidin, monoglucoside, and diglucoside present in the mother-liquor from the original picrate precipitate (X) was made as follows. The anthocyanidin was extracted by means of ether saturated with picric acid, the monoglucoside by carvone-picric acid, and the diglucoside by amyl alcohol-acetophenone (2 : 1 vol.) picric acid solution. In each case the solution of pigment was shaken with the extraction medium until no more colour was removed, the combined extracts were then diluted with ether (light petroleum in the case of anthocyanidin) and shaken out with 0·5 % hydrochloric acid (50 cc.). This aqueous solution was freed from picric acid by extraction with ether and then compared colorimetrically with cyanidin, 3-β-galactosidylpeonidin chloride or mecocyanin chloride. The solution was found to contain 0·03 mg. anthocyanidin, 0·21 mg. monoglucoside, and 0·07 mg. diglucoside per cc. This procedure is due to (Willstätter and) Schudel [1918].

Examination of the anthocyanin. The crude chloride (0·8 g.) was dissolved in warm 5 % ethyl alcoholic hydrogen chloride, but crystallisation did not occur on cooling, and the solution was kept in a vacuum in a vessel containing ether. The alcohol was gradually diluted with ether and a poor type of crystallisation ensued. The indefinitely crystalline crust was then triturated with 5 % alcoholic hydrogen chloride and, although a large loss resulted, an insoluble material was obtained which could be crystallised by dissolution in 0·5 % hydrochloric acid and addition of 5 % ethyl alcoholic hydrogen chloride, after

which the solution was kept in the ice-chest. A recrystallisation in the same way furnished rectangular, prismatic needles, deep-red by transmitted light under the microscope and almost black in mass. (Found in air-dried material: C, 47·9; H, 5·4; Cl, 6·5; loss at 105° in a high vacuum over phosphoric anhydride, 7·4, 7·9. $C_{22}H_{23}O_{11}Cl$, $3H_2O$ requires C, 47·8; H, 5·3; Cl, 6·4; $2H_2O$, 6·5. Found in the material dried at 105°: C, 51·0; H, 5·2; Cl, 6·6; MeO, 6·1. $C_{22}H_{23}O_{11}Cl$, H_2O requires C, 51·2; H, 4·8; Cl, 6·8; MeO, 6·0 %.)

The synthetic 3-glucosidylpeonidin chloride contains $2H_2O$, both molecules being evolved at 110° in a high vacuum. Little importance is, however, attached to this divergence, since it has been the repeated experience of workers in the anthocyanin field that the degree of hydration of the salts can be greatly affected by small concentrations of foreign substances.

Hydrolysis to the anthocyanidin. The last two alcoholic mother-liquors contained the major part of the pigment, and the alcohol was removed by distillation; the residue was then heated on the steam-bath until a test with amyl alcohol showed that the anthocyanin had all been hydrolysed. Care was taken not to heat any longer than was necessary. The anthocyanidin was converted into picrate and this was crystallised from methyl alcohol and then transformed into the chloride by means of methyl alcoholic hydrogen chloride and ether. The precipitated chloride was well dried and crystallised by dissolution in hot ethyl alcohol and addition of one-third of the volume of 12 % hydrochloric acid. It separated in prisms having a green reflex and appearing orange-brown by transmitted light under the microscope. (Found: C, 52·5; H, 4·7; loss at 105° in a high vacuum, 7·4. $C_{16}H_{13}O_6Cl$, 1·5 H_2O requires C, 52·8; H, 4·4; 1·5 H_2O, 7·4. Found in anhydrous material: C, 57·2; H, 4·2; MeO, 9·4. $C_{16}H_{13}O_6Cl$ requires C, 57·1; H, 3·9; 1MeO, 9·2.)

Peonidin chloride synthesised by Murakami and Robinson [1928] was crystallised in the same way and with the same results (1·5 H_2O), but this is the first time that the prism-crystals have been obtained with peonidin from natural sources. The very small amount of the pure peonidin chloride that remained was employed for a study of the colour reactions in buffered solutions of graded p_H. There was only sufficient for twelve tests and the solutions numbered (2)–(13), inclusive in the memoir of Robertson and Robinson [1929], were employed; the results with these twelve solutions agreed completely with the description there given for peonidin.

Properties of oxycoccicyanin chloride and comparisons with 3-β-glucosidylpeonidin chloride. The anthocyanin is rather readily soluble in 0·5 % hydrochloric acid; much less readily soluble in 5 % hydrochloric acid to solutions which become very much more blue-red on dilution with alcohol. Its solution in aqueous sodium carbonate is rich violet in colour and there is no change on the addition of sodium hydroxide, except that the colour soon fades becoming greenish yellow.

The *absorption* of light in the visible region was identical with that of a specimen of 3-β-glucosidylpeonidin (see Fig. 1). The slight divergence from

3-β-galactosidylpeonidin is not significant and probably means that the galactoside was exceptionally pure.

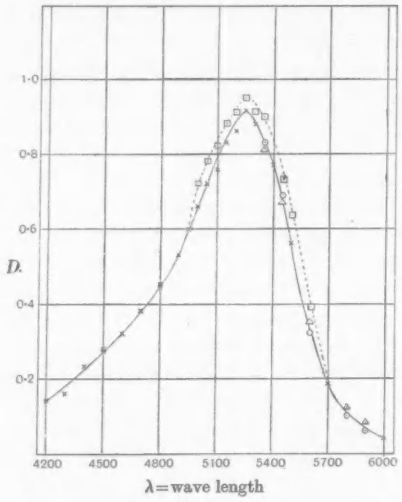


Fig. 1. ⊙ 3-β-Glucosidylpeonidin chloride (natural).
△ 3-β-Glucosidylpeonidin chloride (synthetic).
⊡ 3-β-Galactosidylpeonidin chloride.
× Coincident points.

The *distribution number* was determined using 0·5 % hydrochloric acid (50 cc.) and amyl alcohol (50 cc.). Using 2·432 mg. of the anthocyanin, 26·6 % passed into the alcoholic layer; a second shaking extracted 28·5 % of the material remaining in the aqueous solution. Using 4·907 mg. the corresponding figures were 21·4 % and 22·3 %. These figures did not agree as sharply as we anticipated either with the synthetic glucoside or synthetic galactoside.

However, we had previously tested the crude anthocyanin against glucosidylpeonidin and found identity, so that we were inclined to suspect that the conditions were in some way not quite standard. Using exactly the same conditions, that is the same actual samples of 0·5 % hydrochloric acid and amyl alcohol, we found that with synthetic 3-β-glucosidylpeonidin chloride (5·017 mg.) the distribution numbers for the first and second shakings were 21·9 and 22·6 respectively.

Colour reactions in buffered solutions. The solutions employed were those specified by Robertson and Robinson [1929], and oxycoccicyanin chloride, 3-β-glucosidylpeonidin chloride and 3-β-galactosidylpeonidin chloride gave identical results, except that a few notes on very slight and probably non-significant divergences of the galactoside were made. (2) salmon pink, rapidly fading; (3) similar, fading more rapidly; (4) a little bluer, also fading;

(5) cherry red, much more stable; (6) bluer, more intense, more stable; (7) similar but brownish tinge; (8) a shade bluer; (9) similar, dull red-violet; (11) sharp change to deep violet; (13) the same; (450 cc.), the same but rapidly fading to smoky colour and then yellow. After 1 hour: (2), (3), (4) and (5) formed a decreasing faded series; (6) faded, but intensity between (2) and (3), the galactoside a little the more stable; (7), (8) faded brown-violet; (9) bluer and more intense; (11), (13) violet, little changed; (450 cc.), yellow. Hydrochloric acid added to (4) and (5) restored the colour quantitatively. After 24 hours the solutions in the three series were identical: (2) pink; (3) nearly colourless; (6) colourless; (7), (8), (9) gradual increase to smoky violet; (11), (13) good violet; (450 cc.) yellow.

The identical colour reactions of chrysanthemin chloride and idaein chloride (synthetic specimens) have also been observed by the standard method. (1) salmon red; (2) a little bluer; (3) a little bluer, fading most rapid here; (4) cherry red of greater tinctorial intensity; (5) deeper, fades more slowly; (6) still deeper; (7) duller and browner; (8) still browner; (9) brown-red; (10) the same; (11) the same; (12) the same; in the series (9)–(12) we thought idaein had a little the greater intensity; (13) brown-violet; (14) reddish violet; (15) violet, (13)–(15) lose the blue tinge on keeping; (16) much bluer violet; (17) pure blue fading to greenish blue and then yellow. After 24 hours the 2 series were identical: (1) salmon red; (2) weak bluish red with small precipitate; (3), (4) nearly colourless with violet precipitate; (5) weak violet and precipitate; (6) violet, no precipitate; (7), (8), (9), (10), (11) increasing series to violet-red; (12), (13) a little weaker colour than (11); (14) much weaker violet-red; (15), (16) weak yellow; (17) more intense yellow.

The authors wish to thank the National Research Council (U.S.A.) for a Fellowship awarded to one of them.

Summary.

The fruits of *Oxycoccus macrocarpus* Pers. have been found to contain a pigment which has been isolated as the chloride and identified as a 3-glucoside of peonidin by analysis and comparison with a synthetic specimen.

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CLXXXIV. THE ERGOTHIONEINE CONTENT OF THE BLOOD IN HEALTH AND DISEASE.

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(*Received August 27th, 1931.*)

It is now definitely established that ergothioneine is present in the corpuscles of human blood and of certain animal bloods. Hunter and Eagles [1925] isolated the compound from pig's blood and later [1927] referred to its isolation from children's blood. Benedict, Newton and Behre [1926] also isolated the substance from both pig's and human blood. Hunter [1928] has briefly surveyed the history of ergothioneine and has pointed out the importance of two delicate colour tests to which the substance responds, the development of a blue colour with the uric acid reagents and a new reaction developed by Hunter from the Ehrlich diazo-reaction. Methods for the estimation of ergothioneine based upon the colour reaction with the uric acid reagents were used by Benedict *et al.* [1926, 1929, 1, 2], and by Rockwood, Turner and Pfiffner [1929]. In the earlier publication of Benedict, the following quantitative data were given:

Normal human blood	14–15 mg. ergothioneine per 100 cc.
Diabetic ,, ,,	20–27 mg. ,, ,, ,,
Nephritic ,, ,,	Normal or low values.

However, in Benedict's later publication, the above figures were stated to be too high. A new normal average for human blood was given as 7·5 mg. per 100 cc. whole blood (range 4·2–15·0), and apparently no further study of pathological cases was made.

Rockwood *et al.* [1929] show values for the ergothioneine content of various pathological bloods "in terms of uric acid." The factor of conversion of "ergothioneine in terms of uric acid" to actual ergothioneine was not determined or used by them for the particular method of estimation employed. It thus appeared that more reliable information concerning the ergothioneine content of pathological blood would be desirable. The work fell into two sections: firstly, the development of a reliable technique for the estimation of ergothioneine, which could be also combined with the blood-uric acid estimation, and secondly, the application of this to a series of blood specimens.

Discussion of certain factors in connection with the estimation of ergothioneine.

Benedict *et al.* [1926] found that, in the preparation of protein-free blood filtrates, some tungstic acid reagents caused a loss of all or much of the ergothioneine. This was confirmed by Eagles and Vars [1928]. As a remedy, Benedict

and Newton [1929, 1] adopted a molybdic acid reagent and later [1929, 2] a tungstomolybdic acid reagent for the removal of blood-proteins. Each of these reagents was stated to cause no precipitation of ergothioneine.

In order to choose a suitable blood-protein precipitant, a series of thirty blood samples (mostly pathological) has been used to determine which of the above three methods of protein removal yielded blood-filtrates with the greatest content of ergothioneine. As both uric acid and ergothioneine contribute to the colour developed in the "direct" uric acid estimation, "direct" uric acid values were compared for this purpose. It was found that using reagents prepared from the samples of chemicals stocked in this laboratory, the tungstomolybdic method caused least loss of ergothioneine, and the molybdic method most loss. The tungstic method was found to be nearly as satisfactory in this respect as the tungstomolybdic method (Table I).

Table I.

	"Direct" uric acid, mg. per 100 cc.		
24 samples of human blood	Tungstic	Molybdic	Tungstomolybdic
Maximum	8·1	—	8·6
Minimum	1·6	1·1	1·6
Mean of 24	3·70	—*	3·75
6 samples of pig's blood			
Maximum	1·5	1·8	2·4
Minimum	0·9	0·6	1·2
Mean	1·17	1·03	1·54

* 14 samples only, estimated by the molybdic method; all the figures were definitely low.

This indicated that the use of tungstomolybdic acid as a protein precipitant would be most desirable in blood-ergothioneine determinations. A support for this conclusion was established by the finding that ergothioneine added to blood could be quantitatively "recovered" from tungstomolybdic filtrates. The ergothioneine used was very kindly supplied by Dr G. Hunter.

Folin claimed [1930] that the whole of the ergothioneine of normal blood is retained within the corpuscles when the unlaked blood extract is prepared. That this is also true for pathological bloods has been shown by an examination of twenty-five blood specimens. In the same experiments it was also shown that the centrifugate obtained in the preparation of unlaked extracts, which contains the whole of the blood-ergothioneine, could be used for the estimation of ergothioneine. The actual method, in an improved form, is described later in this paper.

Table II.

	Uric acid, or "ergothioneine in terms of uric acid," mg. per 100 cc.		
	Unlaked extract		
25 human blood specimens	Uric acid	"Ergothioneine"	Centrifugate, "Ergothioneine"
Maximum	13·3	< 0·2*	1·0
Minimum	1·3	—	0·2

* By the method used for this series, amounts of less than 0·2 mg. per 100 cc. blood could not be estimated. The actual values probably approached zero.

The method of differential determination of uric acid and ergothioneine.

The following reagents were used:

(1) Sulphate-tungstate solution, containing 34 g. crystallised sodium sulphate and 6 g. sodium tungstate per litre of solution.

(2) *N*/3 sulphuric acid.

(3) Tungstomolybdic reagent of Benedict and Newton [1929, 2].

(4) 0·62 *N* sulphuric acid.

(5) Acid silver lactate reagent prepared according to Folin [1922]. The solution was always filtered immediately before use.

(6) 10 % NaCl in 0·1 *N* HCl.

(7) Cyanide-urea reagent of Folin [1930].

(8) Folin-Marenzi uric acid reagent [1929].

(9) Standard uric acid solution of Folin [1930].

4 cc. oxalated non-haemolysed whole blood were added to 32 cc. sulphate-tungstate solution. After standing with occasional gentle rotatory shaking for 15 minutes, 4 cc. *N*/3 sulphuric acid were added slowly and with shaking, and the whole mixture was centrifuged as completely as possible. The supernatant liquid was poured off completely and used for the determination of uric acid by the method of Folin [1930].

The centrifugate obtained was transferred quantitatively with small amounts of water and repeated shaking to a long narrow test-tube graduated at 32 cc. about half-way up. The volume was made up with water practically to this mark and the froth removed by means of a trace of capryl alcohol. The volume was then made up to 32 cc. and the contents of the tube were mixed by closing with a bung and inverting several times, avoiding further frothing. This process was found completely to lake the corpuscles. 4 cc. tungstomolybdic reagent were then added and the contents of the tube mixed again, this effecting re-solution of much of the precipitated plasma-proteins, and causing a colour change from opaque red-brown to a clear deep red. The whole mixture was then poured completely into a wide-necked flask, adequate time being allowed for drainage. 4 cc. 0·62 *N* sulphuric acid were then added slowly and with shaking to precipitate the whole of the protein, after which the mixture, occupying a total of 40 cc., was filtered through a 9 cm. No. 40 Whatman paper.

20 cc. filtrate were almost always available and were transferred to a round-bottomed cylindrical centrifuge tube. If 20 cc. were not available, some other aliquot portion, say 18 cc., was used and a correction applied in the calculation. To the filtrate, 7 cc. acid silver lactate solution were then added, and, after standing for a few minutes in the dark, the precipitate was centrifuged. The supernatant liquid was discarded and 1 cc. acid sodium chloride added to the precipitate, which was then thoroughly extracted by triturating with a rounded glass rod. The rod was washed down with about 4 cc. water and the precipitate again centrifuged. The supernatant liquid, containing any uric acid

present, was discarded and the extraction with sodium chloride and water repeated. Finally, 5 cc. cyanide-urea reagent were added to the precipitate which was dissolved by stirring, and the solution was washed quantitatively into a large hard glass test-tube graduated at 14 cc. and water added to this mark.

As a standard, 2 cc. dilute "blood-uric acid" standard solution (containing 0·008 mg. uric acid) were transferred to a similar test-tube, and 5 cc. cyanide-urea reagent and 7 cc. water added.

To both standard and unknown, 1 cc. uric acid reagent was then added and mixed. After allowing the tubes to stand for 4 minutes at room temperature, they were heated in a boiling water-bath for 2 minutes and then rapidly cooled in running water. The colours were compared in the colorimeter, without further dilution of the liquid. The unknown solution was set at 20 mm. and the standard colour matched against it. The calculation was made from a graph plotted for the particular reagents used. This was found necessary, mainly on account of the variable "blank" colours yielded by different samples of sodium cyanide. For the estimations here recorded, the following graph was obtained.

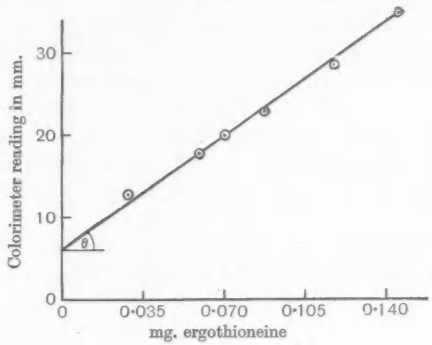

Fig. 1.

The graph is produced back to cut the ordinate at a point representing 6·0 mm., *i.e.* the equivalent of the "blank" colour. The slope of the line, $\tan\theta = 0{\cdot}7$.

If c be the unknown concentration of ergothioneine in a test portion,

y be the reading of the standard colour, matched against the unknown set at 20 mm.,

a be the value in mm. represented by the "blank" colour (constant),

then
$$c = \frac{y - a}{\tan\theta}.$$

This actually reduces to
$$c = \frac{y - 6}{200} \text{ mg.}$$

In the estimation of blood-ergothioneine, the test portion contains the ergothioneine of 2 cc. blood, if 20 cc. filtrate are used, hence

$$\text{mg. ergothioneine per 100 cc. whole blood} = \frac{y-6}{200} \times \frac{100}{2} = \frac{y-6}{4}.$$

The standard colour was found to be stable for only about 1 hour, and hence was conveniently prepared for each ergothioneine estimation. The use of uric acid as a standard was found to be quite suitable, while various dyes were unsatisfactory. The use of pure ergothioneine would necessitate a demand for this substance which cannot as yet be met.

Glutathione and cysteine present in blood in normal amounts exert no interfering effect upon the accuracy of ergothioneine determinations by this method. This has been shown by experiments in which ergothioneine was estimated in blood, with and without the addition of these substances. Finally, the method was found to yield satisfactory duplicate figures.

The findings for normal and pathological blood.

As the ergothioneine of the blood is confined to the corpuscles and as the percentage volume of corpuscles varies, particularly in pathological cases, the corpuscle volume was determined for each blood sample. Uric acid and ergothioneine were estimated by the method described above, the figures being obtained as mg. per 100 cc. whole blood. The ergothioneine was then calculated from the corpuscle volume, as mg. per 100 cc. corpuscles. The results of 60 estimations are shown in Table III. For each group, the figures are arranged in order of increasing ergothioneine content. Blood-sugar values are included for the diabetic bloods and urea values for most of the other pathological samples.

The most striking feature about the results is the large range of ergothioneine values in all the human blood groups studied. The normal range (3–12 mg. per 100 cc. corpuscles) agrees well with that given by Hunter [1928], using an entirely different method of estimation. This range is such as to obscure any variation from the normal due to pathological conditions, and to yield a mean figure of little significance. There is no evidence of any constant variation of the blood-ergothioneine content with sex or with age. Eagles and Vars [1928] have shown that the dietary history of pigs determines the blood-ergothioneine content. However, it is considered that normal human subjects, such as were studied, would be receiving a mixed and balanced diet, without any undue amount of maize protein or other material rich in ergothioneine-producing substances.

In the diabetic series the ergothioneine figures certainly tend to be slightly higher than the normal, but again the range of values is so great as to make this variation of questionable significance. There is no correlation between the ergothioneine and sugar values; neither was any variation in the ergothioneine value related to any stage of the disease or to the treatment. However, this point merits more detailed study.

Table III.

(*a*) *Normal blood.*

Specimen	Age	Sex	% volume of corpuscles	Uric acid, mg. per 100 cc. blood	Ergothioneine, mg. per 100 cc. corpuscles
B.	Adult	F.	47	2·80	3·05
Mixed	,,	—	40	2·92	5·60
C.	,,	M.	49	2·40	6·70
M.	,,	M.	46	3·67	6·85
A.	,,	F.	45	2·75	11·60
D.	,,	F.	51	3·64	11·60
L.L.	,,	F.	45	3·15	12·00
S.S.	11	M.	42	2·93	3·55
B.L.	10	M.	43	2·65	4·05
D.B.	3	M.	37	1·73	6·90
G.H.	4	M.	39	3·32	8·20
Mean of normal samples			... ...	2·85	7·28

(*b*) *Diabetic blood.*

						Sugar, mg. per 100 cc. blood
J.G.	65	F.	46	2·95	6·95	263
E.J.	25	M.	41	2·39	8·15	220
W.	Adult	F.	44	2·38	8·30	180
V.H.	26	F.	45	2·25	8·55	340
C.	Adult	F.	46	1·50	8·80	136
E.B.	23	F.	51	2·52	8·90	250
A.B.	29	F.	45	2·33	9·00	347
A.K.	70	F.	50	3·17	9·30	144
E.H.	61	F.	48	3·39	10·10	208
F.C.	25	M.	53	1·51	10·60	76
W.M. (*a*)	39	M.	41	3·20	11·10	109
N.S.	15	M.	42	2·92	14·05	260
W.M. (*b*)	39	M.	33	3·33	15·15	111
P.L.	10	M.	40	2·94	7·90	170
G.H.	8	M.	41	1·86	9·50	98
R.W. (*a*)	Child	M.	31	5·13	11·95	200
R.W. (*b*)	,,	M.	28	5·16	14·65	266
Mean of diabetic samples			... ...	2·85	10·17	

(*c*) *Nephritic blood.*

						Urea, mg. per 100 cc. blood
M.M. (*a*)	12	M.	29	1·72	4·85	129
B.R.	8	F.	49	2·13	4·90	81
M.M. (*b*)	12	M.	44	1·75	5·25	42
B.H.	7	M.	42	2·63	5·70	80
D.C.	6	M.	56	5·06	5·70	65
I.W.	10	F.	36	4·19	5·85	45
B.G.	12	F.	40	2·94	6·00	59
T.H. (*a*)	12	M.	33	2·92	6·35	54
O.K.	6	F.	29	1·98	7·40	39
T.H. (*b*)	12	M.	46	2·20	7·80	47
G.T.	6	F.	33	2·29	8·20	22
C.W.	5	F.	42	1·82	8·55	42
K.C.	2	M.	21	3·50	10·00	84
M.R.	10	F.	32	2·67	14·55	35
Mean of nephritic samples			... ...	—	7·78	—

Table III (*continued*).

(*d*) *Various pathological bloods.*

Specimen	Age	Sex	% volume of corpuscles	Uric acid, mg. per 100 cc. blood	Ergothioneine, mg. per 100 cc. corpuscles	Urea, mg. per 100 cc. blood
Chorea	10	F.	44	0·97	4·00	33
Prostatitis	55	M.	44	2·59	4·00	40
?	37	M.	46	3·64	4·55	40
Chorea	12	M.	37	1·51	5·00	—
?	57	F.	40	3·69	5·75	67
?	47	M.	25	2·40	6·00	—
Coeliac rickets	7	F.	37	3·43	6·75	76
?	Adult	M.	50	6·56	8·00	—
? Renal tumour	11	M.	48	3·08	9·15	—
Pyelitis	9	F.	46	1·78	9·80	48
Rheumatism	8	M.	43	1·65	10·25	40
Pulm. T.B.	10	F.	45	2·32	13·75	28
Hemiplegia	67	F.	50	3·85	14·70	—
Mean of miscellaneous pathological samples ...				—	7·82	—

(*e*) *Pig's blood.*

1	—	—	43	0·36	15·60	—
2	—	—	50	0·35	20·00	—
3	—	—	45	0·37	21·10	—
4	—	—	36	0·32	22·80	—
5	—	—	55	0·34	24·20	—
	Mean ...	...	... 46	0·35	20·74	—

In the nephritic series the ergothioneine values are normal and do not vary with the retention of nitrogenous excretory products as evidenced by the uric acid and urea figures. A similar large range of values is shown for thirteen miscellaneous pathological blood samples. On the other hand, four out of five specimens of pig's blood, obtained from the abattoir, yielded ergothioneine values substantially higher than those of human blood.

The uric acid figures are of interest as they were obtained by the new method of Folin [1930], for which normal figures have not yet been fully established. In other experiments with both normal and pathological human blood the author has found that normal figures by this new method are consistently slightly lower than those obtained by the older "direct" technique of Folin [1923] for the same blood.

Summary.

A new method for the differential determination of uric acid and ergothioneine in blood is described. Figures are recorded for the uric acid and ergothioneine contents of normal and certain pathological bloods.

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CLXXXV. THE COLORIMETRIC DETERMINATION OF URIC ACID IN URINE.

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The colorimetric method of estimating uric acid in urine has not received attention in the same way as the method for estimating uric acid in blood. Although one of the difficulties of the blood-uric acid determination, namely the development of turbidity in the final solution used for colorimetric comparison, has been entirely overcome, the urine-uric acid method has not been studied, so far as is known, with a view to overcoming this difficulty of cloudy solutions. The Folin method of estimating uric acid in urine [1923] invariably gives turbid final solutions. It has been found always necessary to centrifuge before making the colour comparison. This procedure is satisfactory, except that frequently the turbidity is not completely removed, and even where clear solutions are obtained a fresh clouding often develops while the comparison is being made. Accordingly, the effect of substituting the improved uric acid reagents in Folin's method for urine-uric acid has been investigated and the conditions of reaction modified so that the final solutions are perfectly clear.

Briefly, Folin's method depends upon the reduction of phospho-18-tungstic acid by uric acid in the presence of a large excess of sodium carbonate. Sodium cyanide is also present and the reaction is allowed to proceed at room temperature.

Experimental.

The following reagents were employed.

(1) Uric acid reagent of Folin and Marenzi [1929].

(2) Uric acid standard solution of Folin [1930] containing 1 mg. uric acid per cc. This was diluted ten times with water alone, to prepare the working standard when required.

(3) Cyanide-urea reagent of Folin [1930].

(4) 15 % aqueous sodium cyanide.

(5) Acid silver lactate reagent prepared by the method of Folin [1922]. The solution was always filtered immediately before use.

(6) Sodium carbonate solution containing 20 g. anhydrous sodium carbonate per 100 cc. solution. This is referred to as "20 % sodium carbonate." Suitable dilutions were made to prepare other strengths, *e.g.* "10 % sodium carbonate" (1 : 1 dilution).

The influence of the concentration of sodium carbonate upon colour development and formation of turbidity.

5 cc. portions of dilute standard uric acid solution (each containing 0·5 mg. uric acid) were transferred to each of a series of eleven 100 cc. graduated flasks. To each was added 20 cc. sodium carbonate solution of different concentrations as indicated below. 2 cc. 15 % sodium cyanide were then added to each flask, followed by 5 cc. uric acid reagent. The contents of the flasks were mixed and, after standing for 5 minutes at room temperature, diluted to 100 cc. with water. The turbid solutions were then quickly centrifuged and the colours compared in the colorimeter, using the first solution as a standard set at 20 mm.

Table I.

% concentration of sodium carbonate used	20·0	17·5	15·0	12·5	10·0	7·5	5·0	2·5	2·0	1·5	1·0
Colorimeter reading in mm.	20·0	19·0	18·3	16·6	15·5	14·7	12·8	11·5	11·6	19·9	44·7
Precipitate formation	++	++	++	+	+	±	–	–	–	–	–

It is seen that a reduction in the concentration of sodium carbonate used from 20 % to 2·5 % causes a steady increase in the amount of colour developed. However, the formation of precipitate is only just avoided at the 5 % concentration.

The experiment was then repeated exactly, except that 5 cc. cyanide-urea reagent were substituted for the 2 cc. 15 % aqueous sodium cyanide. The results of this series are below.

Table II.

% concentration of sodium carbonate used	20·0	17·5	15·0	12·5	10·0	7·5	5·0	2·5	2·0	1·5	1·0
Colorimeter reading in mm.	20·0	18·3	17·4	16·3	15·5	14·3	12·6	10·6	10·9	12·7	26·7
Precipitate formation	Trace	Trace	Trace	Trace	–	–	–	–	–	–	–

Again, the colour developed was progressively greater as the concentration of sodium carbonate used was decreased from 20 % to 2·5 %. The formation of precipitate was very slight in the first four solutions, and entirely avoided in the 10 % concentration and below.

Application of the new technique to urine.

As a result, it was proposed at first to use 20 cc. 2·5 % sodium carbonate and 5 cc. cyanide-urea reagent in the uric acid determination as, under these conditions, no precipitate formed in the final solution, and colour development was at a maximum. This modified technique was applied to urine and the results compared with those obtained by Folin's original method.

Two portions of 1 cc. urine were transferred to each of two centrifuge tubes and 3 cc. water followed by 3 cc. acid silver lactate added to each. After mixing and standing in the dark for a few minutes, the tubes were centrifuged and

the supernatant liquid was discarded. One precipitate was dissolved in 2 cc. 15 % sodium cyanide and transferred with 5 cc. water to a graduated 100 cc. flask. The colour was then developed, using 20 cc. 20 % sodium carbonate, and matched against a standard prepared from 5 cc. dilute uric acid standard solution, after centrifuging to remove the precipitate. The result was calculated in grams uric acid per 100 cc. urine. The second silver precipitate was dissolved in 5 cc. cyanide-urea reagent and treated in a similar way except that 2·5 % sodium carbonate was used. The colour comparison was made with an appropriate standard. The type of result obtained is shown in Table III.

Table III.

Specimen	Folin method (15 % NaCN, 20 % sod. carb.)	New technique (Cyanide-urea, 2·5 % sod. carb.)
1	0·064	0·058
2	0·053	0·045
3	0·074	0·061

It is seen that the new method yielded results much below those of Folin's original method, this being apparently due to the reduction in the concentration of sodium carbonate used. Hence, 10 % sodium carbonate was chosen as a possible suitable concentration of alkali. It had already been found that this solution, in combination with the cyanide-urea reagent, yielded the desired colours without turbidity, but at the same time maintained the reaction mixture very strongly alkaline.

Using a number of urine samples, a comparison was made as above between the original Folin method and the new method, using cyanide-urea and 10 % sodium carbonate. Typical results are shown below.

Table IV.

Specimen	Folin's method (15 % NaCN, 20 % sod. carb.)	New technique (Cyanide-urea, 10 % sod. carb.)
1	0·044	0·043
2	0·062	0·063
3	0·051	0·053
4	0·119	0·123
5	0·068	0·067
6	0·051	0·052
7	0·044	0·045
8	0·033	0·033
9	0·074	0·076
10	0·060	0·060
Average	0·0606	0·0615

Agreement between the two methods is here seen to be quite close. In no case was any turbidity produced except when using urines containing less than 0·025 % uric acid. In such a case it is considered that the uric acid reagent is in great excess of the amount needed to react with the uric acid, and this excess is decomposed by the alkali, giving a turbidity. This difficulty was easily avoided by using more than 1 cc. urine in the first treatment with acid silver lactate.

Within limits the new technique was found to give a good degree of proportionality between the uric acid content and colour intensity. This was shown by estimating the uric acid by the new technique in different quantities of the same urine, calculating each estimation to g. per 100 cc.

Table V.

cc. urine taken	1·0	1·5	2·0
Colorimeter reading when standard set at 15 mm.	27·4	17·9	13·8
% uric acid in the urine	0·027	0·028	0·027

It is recommended that uric acid be estimated in urine by the following method.

Transfer 1 cc. urine (or more if the uric acid content is low) to a centrifuge-tube. Add 3 cc. water and 3 cc. acid silver lactate solution. Mix, stand in the dark for a few minutes, then centrifuge. Discard the supernatant liquid. Dissolve the precipitate in 5 cc. cyanide-urea reagent and transfer quantitatively to a 100 cc. graduated flask, using successively two portions of 10 cc. 10 % sodium carbonate solution and finally 5 cc. water.

Prepare a standard by transferring to a 100 cc. graduated flask 5 cc. uric acid standard solution (containing 0·5 mg. uric acid), 5 cc. cyanide-urea reagent and 20 cc. 10 % sodium carbonate solution.

To each flask add 5 cc. uric acid reagent, mix and allow to stand 5 minutes. Dilute to volume, mix and compare in the colorimeter.

SUMMARY.

A modification of Folin's method of estimation of uric acid in urine is described. It is easily carried out in routine practice, and yields results strictly comparable with those obtained by Folin's original technique. It is free from the objection of turbidity in the final colours.

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CLXXXVI. BLOOD-PHOSPHATASES.

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Previous workers [Martland, Hansman and Robison, 1924; Demuth, 1925; Martland and Robison, 1926; Kay, 1929, 1930, 1 and 2] have shown that the red corpuscles and plasma of mammalian blood each contain a phosphatase, but that these enzymes differ in certain characteristics notably in their optimum p_H. Kay [1930, 1] also noted the occurrence of a phosphatase in the leucocytes but made no further study of its properties.

In the present work these phosphatases of the red and white cells and of plasma have been studied and compared with those of other tissues, especially of bone and kidney. It is probable that the phosphatase of the red cells participates in the mechanism of glycolysis in blood [Roche and Roche, 1929] and it was therefore considered that further knowledge of the hydrolytic and synthetic powers of this enzyme was desirable and might help towards understanding the glycolytic process.

Experimental.

The cells were separated from the serum in defibrinated blood or from the plasma in oxalated blood by centrifugation and were washed with a 0·9 % solution of sodium chloride. The upper layer, consisting largely of leucocytes, was removed by pipetting, suspended in isotonic saline and again centrifuged at slow speed. By repeating this process three or four times, the leucocytes were obtained free from red cells. Similarly, by pipetting the lower layer of cells and repeating the centrifugation, the red cells were obtained practically free from leucocytes.

For the enzyme tests, the red cells were laked by adding an equal volume of distilled water and allowed to remain at room temperature for 8 to 10 hours, so that the major portion of the blood-phosphoric esters became hydrolysed. The white cells were lysed with a volume of water equal to the original volume of the blood. One cc. of the lysed cells or of the cell-free serum or plasma was added to 1 cc. of a 0·2 *M* solution of sodium glycerophosphate together with a small drop of chloroform and, after adjustment of the p_H, kept at 37° for 1 hour or longer, the increase in inorganic phosphate being estimated by the Briggs method. The phosphatase content of laked red cells was not appreciably diminished by keeping the solution for periods of 6–10 days at 0° in presence of chloroform. A loss of activity amounting to about 20 % was observed in

one case after 3 weeks at 0°. Blood of rabbits, guinea-pigs and horses was used in these experiments. The red cells of horse blood had a lower phosphatase activity than those of the other animals, but this may possibly have been due to age or condition of the animal.

Attempts to purify the phosphatase of the red cells.

The separation of the phosphatase from the haemoglobin of the laked corpuscles was attempted (*a*) by precipitating the haemoglobin, leaving the enzyme in solution, (*b*) by adsorbing the enzyme on various substances. Precipitation of the haemoglobin with ammonium sulphate yielded a colourless solution, which was, however, completely inactive, both before and after dialysis. Removal of haemoglobin by precipitation as cathaemoglobin with chloroform was more successful. The laked cells were shaken with one-tenth to one-fifth their volume of chloroform and left for some hours at room temperature or 2–3 days at 0°. A considerable proportion of the pigment was thus precipitated, and by repeating this operation three or four times with fresh quantities of chloroform it was possible to remove about 95 % of the haemoglobin with a loss of only 20–30 % of the phosphatase activity. The further removal of the pigment by this method resulted, however, in rapid inactivation of the solution.

Adsorption of the phosphatase was attempted with aluminium hydroxide, kaolin, kieselguhr, barium carbonate and barium sulphate. The first three adsorbents proved satisfactory in so far that they removed a large proportion of the enzyme from the haemolysed cells, but its subsequent elution proved neither easy nor quantitative. This elution was attempted, using water, sodium phosphate solution ($M/15$) and sodium glycerophosphate solution ($M/10$) at various p_H. The best results were obtained with glycerophosphate, but even with this solution less than 20 % of the adsorbed phosphatase was recovered.

These results were not considered sufficiently good to allow the enzyme to be studied in purified condition and the experiments described below were, therefore, carried out using the crude solution obtained by laking the cells.

The optimum p_H *of the blood-phosphatases.*

The optimum p_H of these enzymes was first determined for the hydrolysis of glycerophosphoric ester. The p_H was measured colorimetrically by means of a capillator, a minute drop of the solution being diluted with 10 times its volume of boiled, distilled water in order to reduce the protein error. In judging the p_H of the solutions containing haemoglobin a compensating tube containing the same proportion of pigment but without indicator was used in conjunction with the standards. The results of the first experiments appeared to indicate two definite optima for the phosphatase of the red cells, one near p_H 6 and the second, less pronounced, near p_H 9. The latter was, however, found to be due to the presence of leucocytes mixed with the red cells and was no longer apparent when the cells had been more completely separated.

Table I shows the amount of sodium glycerophosphate hydrolysed by the phosphatases of red and white cells and of serum, at different p_H. In these tests the syrupy 50 % sodium glycerophosphate, which consists chiefly of the α-isomeride, was used.

The figures are calculated for 100 cc. of the lysed cells or of serum.

Table I. *Hydrolysis of glycerophosphoric ester at 37°.*

Red cells		White cells		Serum	
p_H	Hydrolysis in 5 hours mg. P	p_H	Hydrolysis in 8 hours mg. P	p_H	Hydrolysis in 10 hours mg. P
4·0	20·0	4·5	0	4·5	1·2
4·6	25·9	5·3	0	5·5	2·3
5·3	32·2	6·0	1·3	6·0	5·3
5·8	36·7	6·5	6·0	6·5	7·2
6·2	**39·0**	7·3	6·7	6·9	20·3
6·6	34·8	7·9	30·4	7·5	27·2
6·8	23·2	**8·6**	**52·0**	8·3	38·5
7·4	10·5	8·9	48·3	**8·8**	**55·9**
8·3	4·9	9·3	5·1	**9·1**	**55·8**
9·1	0·7	9·6	0·7	9·8	20·0
10·0	0·8	10·2	0	—	—

These results show that the optimum p_H for the phosphatase of the red cells is near 6·2, in agreement with the findings of Martland, Hansman and Robison [1924]; for the phosphatase of serum the optimum p_H is 8·8–9·2, which is the same as the value found by Martland and Robison [1926] and by Kay [1929, 1930, 1], but much higher than that given by Demuth [1925]. The phosphatase of the white cells has an optimum p_H 8·6–8·8, very close to that of the serum. The similarity between the optimum p_H for the phosphatases of serum and leucocytes with that of the bone phosphatase [Robison and Soames, 1924; Martland and Robison, 1927] suggests that these enzymes are probably identical. It is indeed possible that the serum phosphatase may be derived from the bones but, on the other hand, it may partly arise from the leucocytes, some of which are always broken down in blood *in vitro*.

The hydrolytic activity of the blood-phosphatases was next investigated on other substrates, namely, β-glycerophosphate, glucosemonophosphate[1], fructosemonophosphate[2], fructosediphosphate, monophenylphosphate, adenylate and guanylate[3], diphosphoglycerate[4], and the mixed acid-soluble phosphoric esters of the red blood corpuscles. The method of experiment was the same as described above, except that the amounts of the various esters added to 1 cc. of the enzyme solution were equivalent to 1·2–1·7 mg. P, instead of 6 mg. as in previous experiments. The p_H-activity curves for the three phosphatases on these different substrates are shown in Figs. 1–4, while the p_H optima are shown in Table II.

[1] Prepared by Dr R. Robison.

[2] Prepared from hexosediphosphate by Neuberg's method [1918].

[3] Prepared by hydrolysis of yeast nucleic acid and isolated in crystalline condition.

[4] Prepared from horse blood by Greenwald's method [1925].

Table II. p_H *optima of the blood-phosphatases on various substrates.*

	Phosphatase of		
Substrate	Red corpuscles	White cells	Serum
Sodium glycerophosphate	6·0–6·2	8·6–9·0	8·6–9·0
Sodium glucosemonophosphate	5·9–6·1	9·0–9·2	8·8–9·2
Sodium fructosemonophosphate	6·2–6·3	—	—
Sodium monophenylphosphate	5·8–6·0	8·4–8·6	8·4–8·6
Sodium adenylate	6·4–6·5	—	—
Sodium guanylate	6·4–6·5	—	—
Sodium diphosphoglycerate	6·6–6·8	8·8–9·2	8·8–9·2
Sodium fructosediphosphate	6·4–6·7	8·8–9·2	8·8–9·2
Blood-phosphoric esters	6·7–6·8	—	—

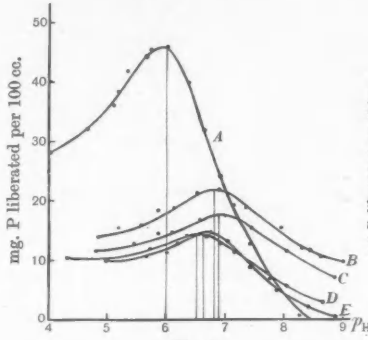

Fig. 1.

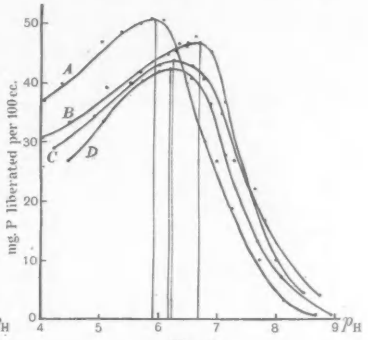

Fig. 2.

Fig. 1. p_H-activity curve for the phosphatase of the red cells. Substrates: *A*, glycerophosphate. *B*, blood-esters. *C*, diphosphoglycerate. *D*, adenylate. *E*, guanylate.

Fig. 2. p_H-activity curve for the phosphatase of the red cells. Substrates: *A*, phenylphosphate. *B*, fructosediphosphate. *C*, fructosemonophosphate. *D*, glucosemonophosphate.

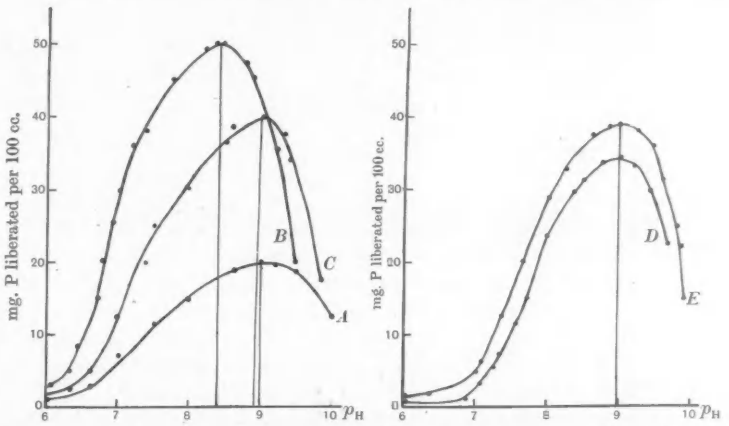

Fig. 3. p_H-activity curves for the phosphatase of the serum. Substrates: *A*, diphosphoglycerate. *B*, phenylphosphate. *C*, glycerophosphate. *D*, glucosemonophosphate. *E*, fructosediphosphate.

It is obvious from these curves that the p_H optimum for each phosphatase varies to some extent with the nature of the substrate. With the enzyme of the red cells it is lower for the monophosphoric ester (5·8–6·4) than for the diphosphoric ester (6·6–6·8). These values are close to that of p_{K_2} (6·72) for

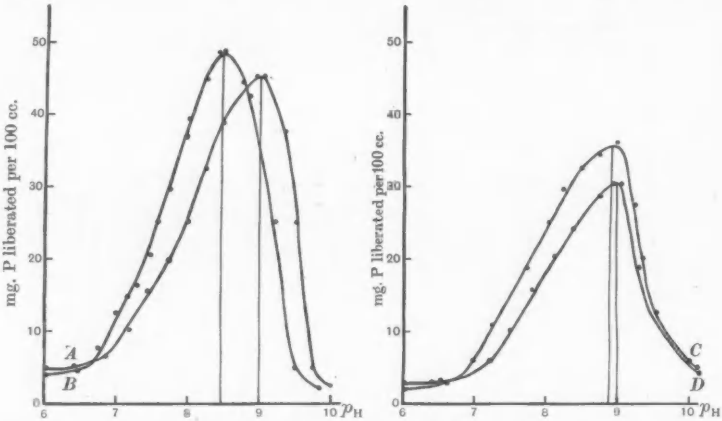

Fig. 4. p_H-activity curves for the phosphatase of the white cells. Substrates: *A*, glycerophosphate. *B*, phenylphosphate. *C*, glucosemonophosphate. *D*, fructosediphosphate.

phosphoric acid and it is possible that the observed differences are related to the differences between the dissociation constants of these esters. That the same optimum p_H was found for the hydrolysis of the blood-esters as for diphosphoglycerate is in agreement with the fact that this substance constitutes the chief part of the acid-soluble phosphorus compounds of the blood.

Such differences in the p_H optima are not so marked in the case of the serum and leucocyte phosphatases but it is possible, as was observed by Martland and Robison [1927] for the bone phosphatase, that spontaneous inactivation of the enzyme occurs at a p_H higher than 8·5, so that the apparent optimum will vary with the duration of the hydrolysis.

Specificity of the phosphatases of the blood and of other animal tissues.

The hydrolytic activity of the blood-phosphatases on various substrates was compared with that of other tissue phosphatases. The results of one typical experiment are shown in Table III. In each series of tests 1 cc. of the same enzyme preparation was allowed to act on an amount of ester equivalent to 1 mg. P at 37° and at the optimum p_H for the enzyme. The duration of the hydrolysis is shown in the Table.

The bone phosphatase was prepared according to the method of Martland and Robison [1929], and p_H 8·8 was adopted, as suggested by them, for tests with this enzyme. Intestinal and kidney phosphatases were prepared by Kay's

method [1926, 1928] and the optimum p_H 8·6, given by this author, was used in the tests.

Table III. *Hydrolysis of phosphoric esters by tissue phosphatases at their optimum* p_H *and 38° (mg. P/100 cc. of enzyme solution).*

	Source of phosphatase					
	Red cells	White cells	Serum	Bone	Intestine	Kidney
Duration of hydrolysis (hrs.)	16	10	16	1·4	1	2
Substrate (sodium salts)						
α-Glycerophosphate	38·8	32·7	27·9	47·1	38·4	15·9
β-Glycerophosphate	11·2	53·4	36·43	69·0	44·4	24·9
Glucosemonophosphate	33·7	58·2	36·9	65·2	37·9	32·8
Fructosemonophosphate	47·1	66·7	44·4	60·4	48·4	40·5
Fructosediphosphate	45·0	67·4	47·2	51·4	47·4	42·4
Monoethylphosphate	12·2	21·0	17·4	16·1	21·3	10·2
Diethylphosphate	0·1	0·1	0·1	0·2	0·2	0·1
Monophenylphosphate	75·6	68·4	63·4	75·2	48·4	39·4
Monopropylphosphate	14·4	19·7	17·1	—	—	—
Dipropylphosphate	0·1	0·2	0·1	—	—	—
Adenylate	8·2	22·1	15·1	22·2	14·0	16·9
Guanylate	9·6	20·4	16·1	25·1	15·2	14·4
Trehalosemonophosphate	5·5	22·1	16·4	20·0	6·1	18·8
Diphosphoglycerate	8·7	17·4	13·7	14·0	9·1	13·3
Pyrophosphate	6·2	31·2	7·0	39·0	35·2	15·2

Action on α- and β-glycerophosphate. Kay [1926] and Kay and Lee [1931] have shown that the kidney and plasma phosphatases attack β-glycerophosphate more readily than the α-isomeride, and the results of my experiments prove the same to be true also for the phosphatases of the leucocytes, bone and intestine and lead me to conclude that this property is common to the various tissue phosphatases for which the optimum p_H is 8·5–9·0. The phosphatase of the red cells, on the other hand, hydrolyses the α-glycerophosphate more rapidly than the β-form. It will be interesting to find whether this holds good also for the muscle enzyme which, according to Kay and Robison [1924], has an optimum p_H close to 7·0.

Action on hexosephosphoric esters. These substrates were readily hydrolysed by all the phosphatases here studied. The hydrolysis of the fructosemono- and di-phosphates was somewhat more rapid than that of glucosemonophosphate except in the case of the bone enzyme, where the relationship was reversed. It is possible that this peculiarity of the bone phosphatase may be due to the relative purity of this enzyme preparation, which contained very little protein.

Action on mono- and di-substituted phosphoric esters. Monophenylphosphate was the most rapidly hydrolysed of all the substrates that were tested. The marked effect of the substituting group on the rate of hydrolysis of the ester is shown by the difference between the results for monophenyl-, monopropyl- and monoethyl-phosphate. Another property common to all the phosphatases was their inability to hydrolyse disubstituted esters such as diethyl- and dipropyl-phosphate. The latter observation is in accordance with the previous findings of Martland and Robison [1927] for bone phosphatase, but in disagreement with those of Neuberg and Wagner [1926], who found that kidney

extract was able to hydrolyse diethylphosphate. This inability to hydrolyse compounds in which two hydroxyl groups of phosphoric acid have been esterified may, as suggested by Martland and Robison, be due to the necessity of two free hydroxyl-groups being present in the substrate in order that it may combine with the enzyme.

Action on other phosphoric esters. The purine nucleotides, trehalosemonophosphate and diphosphoglycerate were hydrolysed much more slowly than the hexosephosphates by all phosphatases.

With pyrophosphate very different rates of hydrolysis were obtained with the different enzyme preparations, which suggests that a specific pyrophosphatase may be present. It may be noted that all phosphatases were found to have some hydrolytic power on the compounds which represent the chief part of the acid-soluble phosphorus of the blood, namely, diphosphoglyceric acid [Greenwald, 1925; see also Macheboeuf, 1927], adenylic acid [Jackson, 1924; Hofmann, 1925], pyrophosphoric acid [Engelhardt, 1930; Roche, 1930] and hexosephosphoric acids [Roche, 1930]. Kay and Robison [1924] found that only a fraction, from 14 to 36 %, of these blood-esters was hydrolysed by the bone phosphatase, a result which may, perhaps, be explained by the relatively slow action of the enzyme on certain of these esters, by the inhibiting effect of inorganic phosphate or by the spontaneous inactivation of the phosphatase during the prolonged experiments.

Factors affecting the rate of hydrolysis of glycerophosphates by the phosphatases of the blood.

Martland and Robison [1927], studying the hydrolysis of glycerophosphate by bone phosphatase, observed that the velocity of this reaction was strongly diminished in presence of inorganic phosphate in concentration as low as 0·005 *M*, while the presence of the second hydrolysis product, glycerol, in concentrations up to 0·1 *M*, had no appreciable effect. Variations in the concentrations of the substrate between 0·003 *M* and 0·3 *M* likewise had no effect on the initial rate of hydrolysis. It would appear that the affinity of the enzyme for inorganic phosphate is greater than for the other constituents of the ester, and it was of interest to find whether this character is shared by the other phosphatases.

The effects of inorganic phosphate and glycerol on the rate of hydrolysis of glycerophosphate (mixed α- and β-forms) by phosphatases of the red cells and of serum are shown in Table IV.

The retarding influence of inorganic phosphate on the rate of hydrolysis is very marked with both these phosphatases, while glycerol also has a considerable inhibitory effect, especially in the case of the red cell phosphatase. The degree of retardation increases with increasing concentration of both hydrolysis products.

Table V shows the effect of variations in the concentration of the substrate on the rate of hydrolysis.

Table IV. *Effect of inorganic phosphate and glycerol on the hydrolysis of glycerophosphate by phosphatases of red cells and serum.*

Initial concentration of ester 0·05 *M*. $t = 37°$. Hydrolysis calculated as mg. P per 100 cc.

Red cells, p_H 6·3	Initial concentration of inorganic phosphate (*M*)					
Hours	*0·03*	*0·015*	*0·01*	*0·005*	*0·003*	*0·002*
1½	2·1	3·3	4·5	7·7	9·7	11·1
3	5·2	7·0	7·8	11·3	13·6	16·0
4½	8·5	10·6	11·0	14·6	18·1	20·1
8	13·3	14·6	16·1	20·8	24·7	27·9
Serum, p_H 8·6	*0·025*	*0·013*	*0·007*	*0·003*	*0·002*	
4½	5·3	6·7	7·5	8·5	8·9	
10	7·9	9·5	10·4	11·7	14·0	
Red cells, p_H 6·3	Initial concentration of glycerol (*M*)					
	0·1	*0·02*	*0·01*	*0·005*	*0*	
3	6·1	10·5	16·2	17·0	19·1	
6½	11·2	16·0	25·1	26·3	31·5	
Serum, p_H 8·6						
4	1·7	2·2	3·4	5·0	6·1	
8	4·3	4·4	8·1	9·1	11·3	

Table V. *Effect of concentration of substrate on the rate of hydrolysis of glycerophosphate.*

Red cells		Serum	
Concentration of ester (*M*)	Hydrolysis in 2 hrs. at p_H 6·3 and 37° (mg. P per 100 cc.)	Concentration of ester (*M*)	Hydrolysis in 6 hrs. at p_H 8·6 and 37° (mg. P per 100 cc.)
0·0075	1·4	0·006	5·9
0·015	2·9	0·013	6·9
0·030	9·4	0·032	7·0
0·075	23·0	0·064	10·2
0·150	37·0	0·128	11·8
—	—	0·320	14·9

With both enzymes the rate of hydrolysis was increased by raising the concentration of substrate between the limits shown in Table V, but, whereas in the case of the serum phosphatase the effect was comparatively small, with the red cell phosphatase the rate of hydrolysis was nearly proportional to the initial concentration of ester. The difference between the behaviour of the serum and bone phosphatases may be due to the specific characters of these enzymes or to the very different state of purity of the preparations used.

The synthetic action of the blood-enzymes.

The synthetic action of bone phosphatase has been demonstrated by Martland and Robison [1927] and that of kidney phosphatase by Kay [1928]. In Table VI the results of a number of experiments are set out, showing synthesis of phosphoric esters effected by the phosphatases of the red cells and of serum in presence of various alcohols. Sodium fluoride in 1 % concentration had no significant effect on such synthesis.

Table VI. *Synthesis of phosphoric esters by blood-phosphatase.*

Alcohol	Inorganic phosphate (mg. P per 100 cc.) Days 0	2	5	10	18	Synthesis (mg. P per 100 cc.)
Serum phosphatase (p_H 8·5: t. 37°)						
Glycol 45 %	159·2	137·2	116·2	115·4	115·4	43·8
Glycerol 40 %	300·2	300·0	296·9	284·9	273·4	26·8
,,	244·4	238·4	—	219·6	199·8	44·6
,,	125·4	117·2	110·2	106·4	108·4	17·0
Sorbitol (saturated)	157·6	156·3	—	—	152·2	5·4
Dulcitol (saturated)	160·1	159·4	—	—	157·4	2·7
Erythritol (saturated)	158·4	160·1	159·2	158·4	157·7	0·7
Glucose 50 %	138·4	139·2	116·9	110·2	109·4	29·0
,, 50 % + 1 % NaF	134·9	134·2	114·4	109·2	106·4	28·5
Fructose 50 %	115·1	112·4	107·6	100·4	92·4	22·7
,, 50 % + 1 % NaF	112·1	113·4	110·4	113·2	92·2	19·9
Glyceric acid 30 %	220·2	218·2	199·9	194·6	192·9	27·3
Red cell phosphatase* (p_H 6·3: t. 37°)						
Glycol 45 %	158·1	144·2	—	130·4	124·4	23·7
Glycerol 40 %	298·7	304·4	—	294·4	270·8	27·9
,,	239·2	244·4	226·4	194·4	190·6	49·6
,,	128·3	130·2	—	122·1	120·1	8·2
Sorbitol (saturated)	158·4	164·4	—	—	160·2	—
Dulcitol (saturated)	159·7	162·4	—	159·4	159·1	—
Erythritol (saturated)	155·4	160·2	—	159·8	160·0	—
Glucose 50 %	127·2	130·6	124·4	118·4	113·4	13·8
,, 50 % + 1 % NaF	121·1	124·6	120·3	114·4	110·4	10·7
Fructose 50 %	121·4	123·4	120·2	116·4	115·3	6·1
,, 50 % + 1 % NaF	127·4	128·9	126·4	120·2	120·1	7·3
Glyceric acid 30 %	195·1	203·2	196·4	—	193·4	1·7

* The slight increase in inorganic phosphates after 2 days' experiment is probably due to the hydrolysis of blood-esters.

Summary.

1. The phosphatases present in serum and in the red and white cells of the blood have been studied and some of their properties compared with those of the bone, kidney and intestinal phosphatases.

2. The phosphatase of the red cells is quite distinct from all the other enzymes. It differs from them in having an optimum p_H on the acid side of neutrality, 6·0–6·8, according to the substrate, and in acting more energetically on α- than on β-glycerophosphate.

3. The phosphatases of the serum and of the white cells appear to be identical, while the small differences observed between the properties of these enzymes and of the bone phosphatase may probably be attributed to differences in the state of purity of the preparations examined. Kidney and intestinal phosphatases are also probably identical with these.

4. The relative rates of hydrolysis of various substrates by these phosphatases have been measured. All monosubstituted phosphoric esters examined were acted on by each enzyme, but the rate of hydrolysis varied with the nature of the substituting group. Disubstituted esters were not appreciably hydrolysed by any of these phosphatases.

5. The hydrolysis of glycerophosphate by each of the blood-phosphatases is diminished by the presence of inorganic phosphate and also by glycerol.

6. The phosphatases of the serum and red cells are able to synthesise phosphoric esters from inorganic phosphate and various alcohols, including glycol, glycerol, and hexoses.

I wish to express my thanks to the Director of the Lister Institute and to Prof. A. Harden for the hospitality of the Biochemical Department and to Dr R. Robison for his interest and helpful criticism in this work. I wish also to thank the Rockefeller Foundation for the Fellowship which enabled me to carry out this research.

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CLXXXVII. EFFECT OF ADDITION OF SALTS ON THE ISOELECTRIC POINT OF PROTEINS. II.

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Fresh evidence will be given in this paper in support of the opinion that the free charge of protein particles in solution is dependent on the presence of neutral salts and on their concentration; in particular on the valency of their anti- and iso-ions.

It was shown in our previous communication [1931] that, as demonstrated by experiments on flocculation induced by heat or by addition of alcohol, proteins may be discharged at p_H other than that of the isoelectric point by the addition of a critical concentration of electrolyte, termed in our previous paper C_K. Experiments will be described in this paper, showing that at concentrations greater than the critical the protein particles may, at the same p_H, be supercharged, so that protein solutions are obtained in which, at p_H values smaller than that of the isoelectric point, the particles behave as anions, whilst at p_H higher than the isoelectric they behave as cations. The possibility of such supercharging was indicated by Kruyt's [1929] experiments on the action of sodium sulphate on the cataphoretic properties of gelatin at p_H 3·9, as well as by the work of Loeb [1924, 1925].

Experimental.

1. Gelatin.

Gelatin was purified by the method described in the preceding paper, and the flocculation of aqueous solutions of such gelatin, in the presence of 60–70 % of alcohol, was studied as before. Since it appeared probable that supercharging could most readily be achieved by the addition of electrolytes to solutions at a p_H differing little from that of the isoelectric point, hydrochloric acid was added to p_H 3·5–3·8, or sodium hydroxide to p_H 6·5–7·0.

1 % gelatin solutions at p_H 3·5 were treated with an excess (0·001–0·004 M) of potassium ferricyanide, the C_K of which is about 0·0001 M at this p_H. In the presence of alcohol (70 %) gelatin is quantitatively precipitated in the presence of 0·001 M $K_3Fe(CN)_6$, whilst when excess of this reagent is taken, 10–20 % of the gelatin remains in solution. Filtrates from such systems were taken for examination.

Various salts (sodium sulphate, potassium ferricyanide, calcium and magnesium chlorides) were added to these filtrates. The results obtained indicate that gelatin treated with an excess of ferricyanide at p_H 3·5 becomes practically insensitive to the action of multivalent anions, but is readily flocculated by salts giving rise to univalent anions and bivalent cations. Thus ordinarily the C_K of sodium sulphate is less than 0·001 M, and of magnesium chloride greater than 0·05 M, whilst supercharged gelatin is not flocculated by the addition of 0·02 M sodium sulphate, and is coagulated by 0·0005 M magnesium chloride.

Similar experiments were performed on alkaline gelatin solutions, at p_H 6·5–7·0, adding excess of neutralised aluminium chloride (0·002–0·005 M), when a precipitate formed, the bulk of which was increased by the addition of alcohol to 70 %. The filtrate contained gelatin which was sensitive to the action of multivalent anions, but not cations. Further addition of aluminium chloride did not lead to flocculation of the residual gelatin, and the same applies to magnesium chloride, the C_K of which is normally 0·0025 M, whilst in this case flocculation is not obtained using 0·02 M solutions. On the other hand, the C_K of sodium sulphate is now 0·0003–0·0008 M as compared with the normal value of over 0·01 M.

2. Serum-albumin.

A similar sensitisation to the action of cations and desensitisation to that of anions is obtained using serum-albumin solution at p_H 3·5 by the addition of excess ferricyanide (0·001–0·005 M), and, conversely, sensitisation to the action of anions and desensitisation to that of cations can be demonstrated in neutral solutions (p_H 6·5–7·0) by the addition of 0·01–0·02 M aluminium chloride. The results obtained are based on observations of coagulation after heating 10 min. at 100°.

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CLXXXVIII. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

IX. THE SYNTHESIS OF PHENYL SULPHATE AND INDOXYL SULPHATE.

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(*Received September 1st, 1931.*)

The theory that the organic or ethereal sulphates arise from phenols by conjugation with sulphur compounds other than sulphate, oxidation occurring subsequently, rests entirely on negative evidence. In more recent years Rhode and Sherwin have supported this theory and Sherwin has gone further, suggesting that ethereal sulphates are formed only from sulphur-containing complexes of endogenous origin. Rhode [1922] in experiments on rabbits, and Sherwin in collaboration with Shiple and Muldoon [1924] in experiments on a pig failed to show any union between phenols and sodium sulphate administered concurrently. They made use of phenol itself and of certain halogenated phenols. The chief evidence for the older theory [Baumann, 1876], that ethereal sulphates arise by the combination of phenols with preformed sulphate, lies in the ability of the dog to bring about the union of guaiacol and the sulphate ion of sodium sulphate [Hele, 1924, 1]. In discussing these differences Pryde [1931] suggests that "guaiacol is a substance too far removed from the normal metabolic paths to warrant comparison with phenol in this respect." He makes no comment however on differences in animal species or differences in experimental technique.

The pig, unlike the dog, does not utilise sulphur compounds readily in detoxication processes and this may also be true of the rabbit [Coombs and Hele, 1926; Lawrie, 1931]. Under such circumstances to demonstrate the truth of Baumann's theory becomes a matter of extreme difficulty. No one has succeeded in proving it in either rabbit or pig, but failure to do so does not mean proof to the contrary. Apart however from the question of species Rhode and Sherwin with his co-workers, employing phenols of considerable toxicity and in relatively small doses, did not produce experimental conditions that could be successful. In my experiments the dog was used as the most suitable animal and guaiacol was chosen on account of its low toxicity, when given orally in the form of carbonate. The dog, as it were, could be saturated with guaiacol without any effect on its health. All sulphate arising from the tissues or the diet was used up in the detoxication process. The demand for

sulphate was greater than the supply, so that sodium sulphate, added to the diet, was immediately utilised by the animal.

In the present paper evidence is brought forward to show not only that the dog is capable of utilising sodium sulphate in the synthesis of phenyl sulphate and of indoxyl sulphate but also that, when the same syntheses are brought about at the expense of endogenous sulphur, the sulphur so utilised is in the form of the sulphate ion [Hele, 1927].

Experimental.

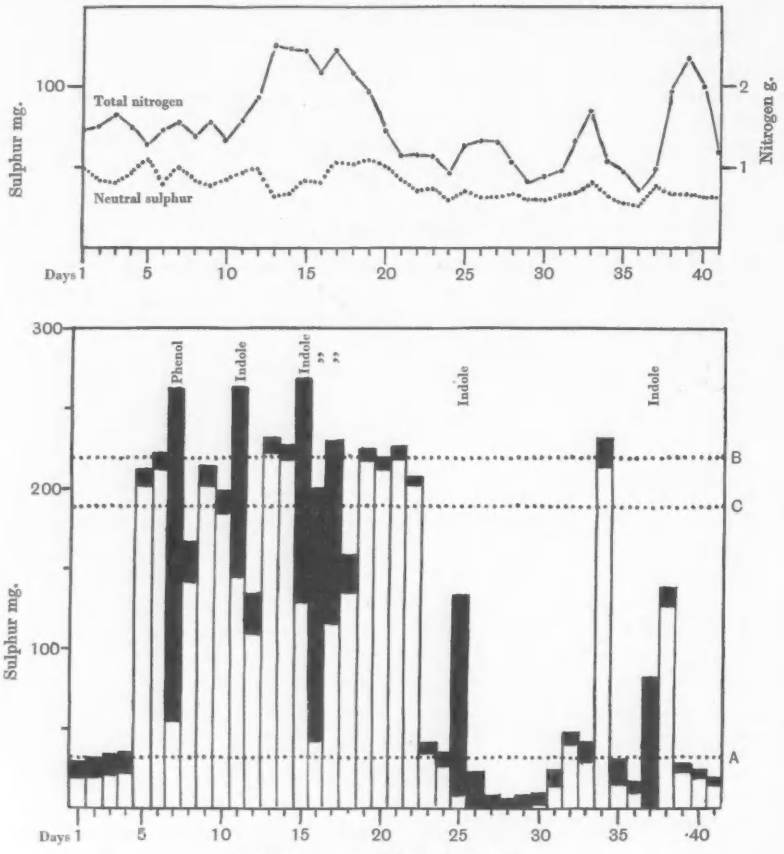
The experimental methods employed in this research have already been described [Hele, 1924, 2]. Two bitches were used, Patsy (6·8 kg.) and Pansy (9·0 kg.). As repeated doses of phenol could not be given safely, the "saturation" method, successfully utilised in the case of guaiacol, had to be discarded. The animals therefore were dosed daily with sodium sulphate and on certain days phenol or indole was administered orally in amounts sufficient to show an effect on the sulphur metabolism. This method of dosage had already successfully demonstrated the capacity of the dog to use sodium sulphate in the formation of chlorophenyl sulphate from chlorobenzene [Hele, 1924, 2]. In order to make the animal dependent for the formation of ethereal sulphate either on its endogenous sulphur or on the sodium sulphate, the protein of the diet and therefore the sulphate, arising therefrom, was reduced to the minimum. The quantity of meat retained in the diet was only just sufficient to place round the gelatin capsules, containing the phenol or indole on dose days. The meat was kept in position round the capsules by a few cotton stitches. Calories were provided in the form of margarine and sucrose. The actual amounts were determined experimentally by the quantities the bitches were prepared to swallow over long periods of time. The number of calories was inadequate and the bitches lost weight. It was found desirable to retain some milk in the diets.

Pansy's diet was as follows:

		Total N g.	Total S g.	Calories K
Meat:	10 g.	0·34	0·023	13
Sugar:	60 g.	—	—	246
Margarine:	50 g.	—	—	400
Milk:	50 cc.	0·26	0·023	36
Water:	100 cc.	—	—	—
	Total	0·60	0·046	695

Patsy received only 40 g. of margarine but in other respects her diet was identical with that of Pansy.

When administered, the daily dose of sodium sulphate was 2 g. of the decahydrate (containing 200 mg. of sulphur), half given at 9.30 a.m., half at 5 p.m., either in the milk of the diet or subcutaneously, dissolved in 5 cc. of water, and the daily dose of phenol or indole was 1/100 of the g. molecular



Black columns = Ethereal sulphate.
White columns = Inorganic sulphate.

Fig. 1. "Patsy."

Days	5–22	2 g. sodium sulphate, cryst. orally, daily.
Day	34	2 g. " " " "
"	38	2 g. " " " "
"	7	1 × M.W./100 g. phenol, orally.
"	11	1 × M.W./100 g. indole, "
Days	15–17	1 × M.W./100 g. " " daily.
Day	25	1 × M.W./100 g. " "
"	37	1 × M.W./100 g. " "

Line A.	Daily sulphate sulphur on diet	32 mg.
" B.	" " " on diet and sodium sulphate	220 mg.
" C.	B less A	188 mg.

Patsy's weight fell from 6·8 to 6·0 kg. in 41 days.

weight (sufficient to combine with 320 mg. sulphur as sulphate), half given orally at 9.30 a.m., half at 5 p.m. The urine was collected from 9 a.m. to 9 a.m.

The first experiment carried out under these conditions was immediately successful. It is shown diagrammatically in Fig. 1. The excretion of total nitrogen and of neutral sulphur is shown in simple curve form. The excretion of sulphate is shown in column form in order to exhibit certain quantitative relations. The black portion of each column represents ethereal sulphate and the white portion inorganic sulphate. The average output of sulphate-sulphur on diet only was 32 mg. Line *A* represents this value. The average output of sulphate-sulphur on sodium sulphate days was 220 mg. (line *B*). On day 7 phenol was given orally and it is manifest that the major portion of the excreted sulphur was in the form of ethereal sulphate. In the formation of ethereal sulphate, it is best to assume that the sulphur first utilised is derived from all sources other than the administered sulphate. Line *C* has therefore been drawn at level 188 mg. S (220 mg. less 32 mg.). The black portion of the column is thus divided into three parts:

(1) a part above line *B* (43 mg. S), in excess of daily sulphate output and therefore derived from the tissues;

(2) a part between lines *B* and *C* (32 mg.), representing the daily output of sulphate derived from the diet and the tissues;

(3) a part below line *C* (133 mg. S), derived from the sodium sulphate.

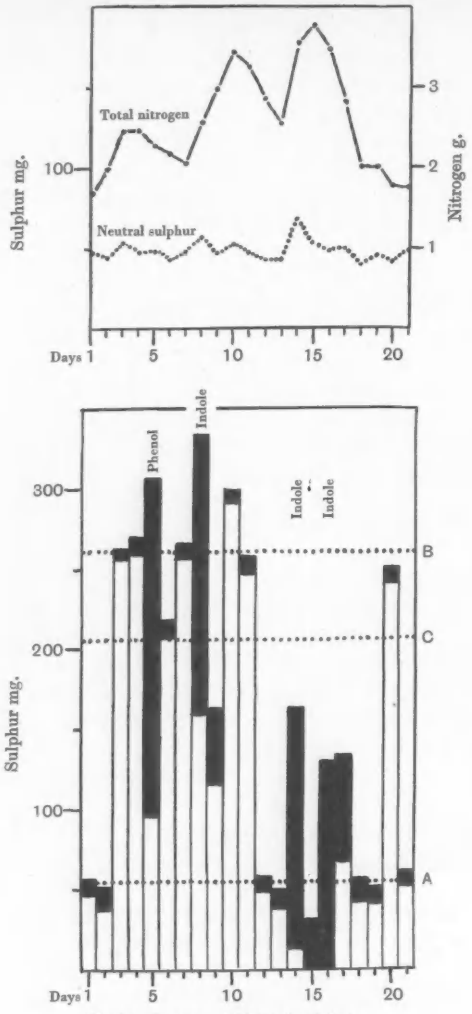
At least 66 % of the administered sodium sulphate has been utilised.

On day 8 the output of sulphate is less. 53 mg. S from some source or other, normally excreted as sulphate, has been retained. The amount retained corresponds in some measure to the excess excreted on the dose day. Presumably the one is the consequence of the other.

A result, similar though not so good, follows on the administration of indole on the eleventh day.

By repeating the daily doses of indole over a period of three days (days 15–17) it was hoped that a greater drain on the endogenous sulphur would result. However there was actually some retention of sulphur on the second day of the series and the net excess sulphur excreted over the 3 days was only 39 mg., a figure very like those obtained for single day doses. It therefore appears that the amount of endogenous sulphur, immediately available for the formation of ethereal sulphate, is limited in amount. The retention of sulphur, following the dose days, was 61 mg.

It was next decided to attempt the replacement of the sulphur lost on dose days by the sulphate of the sodium sulphate. On day 25 indole was administered without sodium sulphate. There was an increased sulphate-sulphur excretion of 102 mg. To make good this loss sulphur was retained for the following 5 days, the total being 103 mg. This must have been derived partly from the food and partly perhaps from the tissues. On day 7 indole was again given alone and on the following day sodium sulphate. On the indole day 50 mg. excess sulphate-sulphur was excreted and on the sulphate

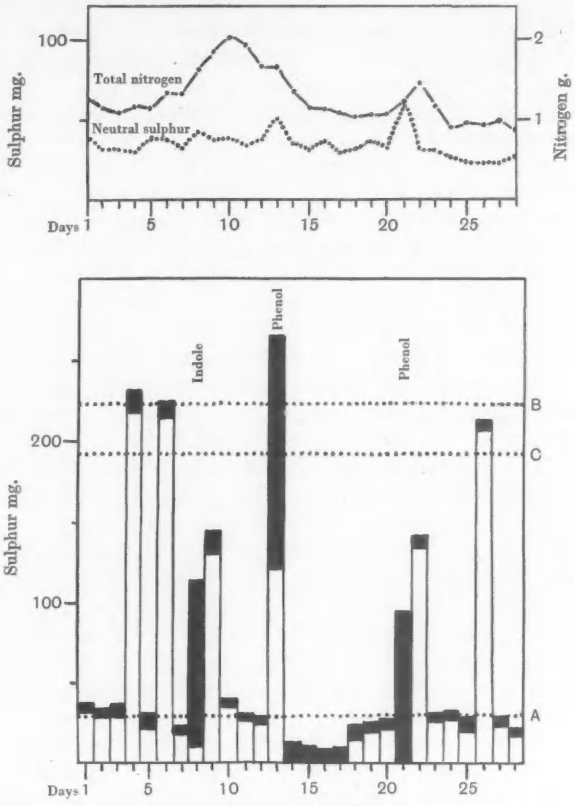


Black columns = Ethereal sulphate.
White columns = Inorganic sulphate.

Fig. 2. "Pansy."

Days 3–11	2 g. sodium sulphate, cryst. orally, daily.	
Day 17	2 g. „ „ „ „	
„ 20	2 g. „ „ „ „	
„ 5	1 × M.W./100 g. phenol, orally.	
„ 8	1 × M.W./100 g. indole, „	
„ 14	1 × M.W./100 g. „ „	
„ 16	1 × M.W./100 g. „ „	
Line A.	Daily sulphate-sulphur on diet	55 mg.
„ B.	„ „ „ on diet and sodium sulphate	262 mg.
„ C.	B less A	207 mg.

Pansy's weight fell from 9·0 to 8·3 kg. in 21 days.



Black columns = Ethereal sulphate.
White columns = Inorganic sulphate.

Fig. 3. "Patsy."

Day 4 2 g. sodium sulphate, cryst. subcutaneously.
„ 6 2 g. „ „ „ „
„ 9 2 g. „ „ „ „
„ 13 2 g. „ „ „ „
„ 22 2 g. „ „ „ „
„ 26 2 g. „ „ „ „
„ 8 1 × M.W./100 g. indole orally.
„ 23 1 × M.W./100 g. phenol „
„ 21 1 × M.W./100 g. „ „

Line A. Daily sulphate-sulphur on diet ... ... 30 mg.
„ B. „ „ „ on diet and sodium sulphate 223 mg.
„ C. B less A ... ... 193 mg.

Patsy's weight fell from 6·8 to 6·5 kg. in 28 days.

day 81 mg. sulphur were retained. Immediately afterwards the sulphate excretion returned to normal. Even if it is assumed that 32 mg. of the sulphur retained were derived from the normal daily output of sulphate, a balance of 49 mg. is left, which must have come from the administered sodium sulphate.

Similar experiments were carried out on "Pansy." Fig. 2 has been constructed on the same lines as Fig. 1. The results obtained by the administration of phenol (day 5) and of indole (day 8) are similar to those obtained in Exp. 1. In the latter case however there are certain discrepancies. The apparent retention of sulphur on day 9 is extremely great and on day 10 the excretion of sulphate is much in excess of the normal. The most likely explanation of these discrepancies is the delayed absorption and therefore the delayed excretion of the sulphate given on day 9. If the excess sulphate on day 10 is added to the sulphate on day 9, the figure for the retained sulphur on that day becomes 62 instead of 100 (see Table I). On days 14 and 16 indole was administered without sodium sulphate, again with the intention of draining the animal of all sulphur available for the synthesis of ethereal sulphate. The excess excretion on the two days was 184 mg. S. Sodium sulphate was administered on day 17. The sulphur retained on days 15 and 17 was 151 mg., of which at least 73 mg. must have been derived from the sodium sulphate.

In Exp. 3 (Fig. 3) the sodium sulphate was given subcutaneously, to eliminate the possibility of any of the sulphate failing to reach the tissues. Indole was given on day 8 and the excess sulphate-sulphur, excreted on this day, was 84 mg. Sodium sulphate was administered on the following day and the sulphur retained on that day was 78 mg. The corresponding figures for phenol (days 21, 22) were 65 and 81. On day 13 phenol was given with sodium sulphate and the excess sulphate-sulphur excreted on that day was 42 mg. The sulphur retained on the four following days was 77 mg. 37 % of the administered sodium sulphate was utilised in the formation of the phenol sulphate. It makes no difference therefore whether the sodium sulphate is given orally or subcutaneously.

The calculations are based on averages for normal diet and normal sulphate days and are therefore subject to error[1]. The nitrogen output in each experiment and to a lesser degree the neutral sulphur output varied most markedly, but in most instances these variations do not seem related to variations in the sulphate output. However, the corresponding variations in the sulphate excretion, if they occur, would be small and would be masked by the much greater variations due to the administration of the sodium sulphate, of the phenol and of the indole. On the whole it is somewhat remarkable that the calculations bear out the main thesis of this paper so consistently as they do.

[1] For example: in Exp. 2 line C is absolutely too high; it is drawn at the level of 207 mg. S and actually only 200 mg. S were given as sodium sulphate on sulphate days.

Discussion.

In calculating the figures, which appear in Table I, two assumptions have been made—that the administered sodium sulphate is the last source of sulphur to be used (1) in the formation of ethereal sulphate and (2) in making good the sulphur lost from the tissues on dose days. The figures given therefore in the Table under sodium sulphate are minimum values.

Table I.

Day	Substance	Percentage phenol or indole excreted on dose day as ethereal sulphate	Ethereal sulphate excreted on dose day: From excess sulphur mg. S	From sulphur of diet mg. S	From sodium sulphate mg. S	Total mg. S	Percentage sodium sulphate utilised	Sulphur retained after dose day mg. S	Minimum amount of sodium sulphate retained mg. S
Exp. 1:									
7	Phenol	62*	43	32	133	208	66	53	21
11	Indole	34*	44	32	43	119	22	85	53
15–17	Indole	40*	39	96	278	413	46	61	29
25	Indole	36*	102	24	—	126	—	103	—
37	Indole	23	50	32	—	82	—	81	49
Exp. 2:									
5	Phenol	63	45	55	112	212	56	43	—
8	Indole	50*	71	55	49	175	25	100	45
14	Indole	41*	109	34	—	143	—	23	—
16	Indole	37*	75	55	—	130	—	128	73
Exp. 3:									
8	Indole	30	84	20	—	104	—	78	48
13	Phenol	43	42	30	73	145	37	77	—
21	Phenol	27	65	30	—	95	—	81	51

* Some in addition was also excreted on the day following the dose day.

It is clear from the Table that 23 to 62 % of the phenol or indole administered has been excreted on the dose day in the form of ethereal sulphate and at least 22 to 66 % of the sodium sulphate given has been utilised in the synthesis of the ethereal sulphate. One cannot expect all the administered sulphate to be utilised. Some will always escape through the kidney, before the synthesis can take place. Also not all the phenol or indole is available for the sulphate synthesis; part is excreted with glycuronic acid and part is probably oxidised. The utilisation of the sodium sulphate has been as full as can be expected. The mode of formation of ethereal sulphate from phenol or indole is the same as that from guaiacol.

In the Table are also shown the supposed sources of origin of sulphur, taking part in the ethereal sulphate synthesis. In column 4 are given the amounts of excess sulphur excreted on dose days. Such sulphur would commonly be called endogenous. Some persons might demur to attaching such a label to small amounts of excess sulphur, amounts that might be regarded as exogenous products delayed in excretion. When however the excess figures become large, it would be difficult to avoid the term endogenous. In the Table the corresponding figures for retained sulphur are also given and in the last column from these have been deducted the normal sulphate output

on diet days. The balance must represent the minimum amounts of sodium sulphate retained to make good the loss of sulphur from the tissues.

The most likely explanation of these experimental facts is the simplest. There is no reason to suppose that there are any differences in this particular respect in the oxidation products of endogenous and exogenous sulphur. It is unnecessary to distinguish between the two lines of metabolism. It is enough to say that sulphate arises by oxidation of sulphur compounds in organs. Before the sulphate is finally excreted by the kidneys, it accumulates in the body fluids. In a small dog this accumulation amounts to about 50 to 100 mg. S.

In the detoxication of phenols the first sulphur to be used is probably the sulphate ion in the cell fluids. As the immediate supply of sulphate is used up, it is replaced by oxidation products of metabolism. When the body fluids are depleted of sulphate by the administration of phenols, sulphate accumulates slowly over a series of days as it arises in the course of metabolism or as it can be replaced by sulphate in the diet, until the concentration is such that excretion again becomes normal. It seems likely that the sulphate excreted in excess on dose days and the sulphate retained afterwards are present in the tissues in the ionised condition and not in combination and undissociated, but of this there is no proof. To some the retention of sulphate by the animal may seem remarkable but it must be remembered that the concentrations involved are no greater than the concentration of sulphate (2 to 3·5 mg. S per 100 cc.) in normal dogs' blood [Denis, 1921]. If the sulphate retained were distributed uniformly (a more than improbable assumption) throughout the body fluids, the concentration would be about 1 to 2 mg. S per 100 cc.

Summary.

1. The dog can utilise sodium sulphate, given orally or subcutaneously, to form phenyl sulphate and indoxyl sulphate from phenol and indole respectively.

2. When ethereal sulphate is formed by the dog from phenol or indole at the expense of endogenous sulphur, this sulphur can be replaced at least in part by the sulphate ion.

3. It is suggested that in every case ethereal sulphate, whether arising endogenously or exogenously, is formed by the union of the sulphate ion with phenolic compounds.

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CLXXXIX. INVESTIGATIONS ON THE DETERMINATION OF VITAMIN D.

COMPARISON BETWEEN THE PREVENTIVE AND THE CURATIVE METHODS.

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Introduction.

For the biological standardisation of vitamin D, several fundamentally different methods have been devised, but only three of these are in use. These methods are based on the presence or absence of rickets in young rats kept on a rachitogenic diet *plus* a varying addition of vitamin D. The preventive method consists in giving the vitamin D addition to the experimental animals at the same time as they are placed on the rachitogenic diet. The curative method implies the supposition that after rickets is established in the animals they are cured of this lesion by a suitable addition of vitamin D to the rachitogenic diet. Not all investigators insist upon complete recovery, however, but merely a certain degree of recovery. In these two methods the decisive point is, whether at the end of the experiment the animals have rickets or not; yet, as mentioned, a slight degree of rickets may be allowed in tests by the curative method. To decide whether rickets is present or to what extent the condition of rickets has improved, the various authors have used different methods. Macroscopic pathological-anatomical examination is the simplest method, but most authors are agreed that this is relatively rough. It may be supplemented by histological examination of the bones, but this complicates the test considerably.

X-ray examination is just as simple as the macroscopic pathological-anatomical examination. It was first employed by American investigators and it has the great advantage that it is practicable on the living animal. Systematic X-ray examination has been elaborated by Poulsson and Løvenskiold [1928], and others. Methods based on chemical analysis are somewhat more circumstantial, whether concerning the dry substance, ash (including Ca and P) of the bones or the calcium and phosphorus content of the serum.

Of these methods of examination it may be said that the investigators working with them have obtained results they consider satisfactory.

Weighing the two methods, it seems obvious to me that the preventive

method offers several advantages over the curative. It takes less time for the experiment. Moreover a "preventive dose" is more precise than a "curative dose," as several authors differ in their requirements as to the time in which recovery, or relative recovery, is to take place.

The third method to be mentioned in this paper is Shipley's line test [McCollum, Simmonds, Shipley and Park, 1922]. In the last two years considerable attention has been paid to this method. In this method it is not the recovery of rachitic young rats that decides the estimate; the essential feature of the line test is a sign of commencing recovery, namely, the precipitation of calcium phosphate, demonstrable with silver nitrate under influence of the light as a blackening around the epiphyseal lines (tibia). The method has been the subject of thorough investigations [Steenbock and Black, 1925; Coward, 1928; Adams and McCollum, 1928; Bills *et al.* 1931], and judging from these, it appears to be serviceable. Still, several objections may be raised to this method—objections which have indeed been foreseen by several of the authors mentioned, without, I think, being sufficiently refuted.

One weighty objection is that the line test may give a positive result in conditions other than commencing recovery. As has been demonstrated first by Cavins [1924], in young rats there appears on inanition a very distinct precipitation of calcium salts at the epiphyseal line. This precipitation is so marked that it can be demonstrated with X-rays. Of course, the vitamin tests in question do not involve any true inanition, but from the papers quoted it is evident that the weight curve of rachitic young rats will often show a marked change when the diet is supplemented with an addition, for instance, of cod-liver oil. Thus, Adams and McCollum give several instances of such an influence upon the weight curve, the animals showing a natural rise of weight during the fore-period and a loss of weight during the test period. Quite naturally, one will think that these instances involve at least a partial inanition. In going through the tables of Adams and McCollum, it is found in keeping with this, that the most positive results of the line test are obtained in the rats that are losing weight. Of course, rats that are losing weight may be left out of consideration, as well as the rats with a strikingly small weight during the test period; and, in fact, Bills *et al.* [1931] recommend such a procedure. But a method that allows of exclusion of test animals according to arbitrary criteria involves a certain degree of unreliability, and should, therefore, be avoided as far as possible.

Nor is there any doubt that a quantitative estimation of the line test is difficult. I am here leaving out the macroscopic estimation and refer to the microscopic examinations reported by Adams and McCollum and by Bills *et al.* They show that in the line test the histological picture is rather polymorphous. The calcium precipitation may take place in several ways. The last scheme worked out by Bills must be said to show a distinct gradation of the precipitation phenomena. But the fact that there is a rather wide scattering in the results of the line test in animals getting the same addition

makes it evident that the significance of this gradation may still be rather problematic; and it is also questionable whether Bills is justified in working with the average of his arbitrary gradation. Besides, as mentioned before, some animals have already been left out on account of their weight.

Experimental.

In the following I have tabulated a number of experiments by the preventive and the curative methods; and I have also tried to include the line test in these comparative examinations. In the preventive method that dose of cod-liver oil is designated as preventive which is the smallest of the doses employed that is sufficient to keep the test animals free from rickets. In the curative method, the curative dose of cod-liver oil is the dose which is just enough to give the greatest effect, while greater doses have no additional effect under the given experimental conditions.

Technique.

The experiments were made on young white rats, 30 days old, taken direct from the mother. The stock of breeding animals has been kept through 10–11 generations on Gudjónsson's diet No. 4 [1930], so there have been but comparatively slight variations in the size of the litters and the average weight of the young.

Gudjónsson's diet No. 4 consists of:

Skimmed-milk powder	30 %
Rice flour	40 %
Autolysed yeast	15 %
Coconut oil (with 0·3 % shark-liver oil) ...	15 %

The young rats are transferred from the stock quarters to the experimental room, where daylight is always shut out. The temperature of this room has been recorded by means of a thermograph, and throughout all the experiments it has been 20–25°. Each animal has been alone in its cage. In all cases the diet in the experimental period has been McCollum's diet No. 3143, which consists of:

Whole wheat	33 parts
Whole corn (yellow)	33 ,,
Wheat gluten, powdered ...	15 ,,
Gelatin, powdered	15 ,,
Calcium carbonate	3 ,,
Sodium chloride	1 part

The wheat was a mixture of American and Danish wheat (Havnemöllen Ltd., Copenhagen). The yellow corn was La Plata corn (Ballerup Mill Ltd., Copenhagen). The wheat gluten was prepared from a mixture of American and Danish wheat (Leerbeck and Holm, Copenhagen). The gelatin was the

brand V.I. (T. M. Duché and Sons, London). The inorganic salts were chemically pure (*pro analysi*).

All additions of cod-liver oil were given separately in a glass dish as solutions in peanut oil, so that in each case the addition has been 0·20 g.

In this laboratory we prefer to give the addition as mentioned rather than mix it with the food or put it directly into the mouth of the animal. We find it more convenient and just as reliable as the last-mentioned procedure, as the animals devour it completely at once.

X-ray examination. When the animals are entered in the tests, X-ray examination of the left tibia is made at the same time, without anaesthesia. By means of a rubber band the animal is fastened in a wooden box in such a manner that the projection is the same in all the exposures. The X-ray examination is carried out with a Wappler X-ray dental apparatus. The time of exposure is $\frac{1}{4}$ second at 15 cm. with a current of 15 milliamperes and a tension at the tube of *ca.* 50 kilovolts. In judging the X-ray findings importance has been attached to the width of the epiphyseal line and to the density of the metaphysis. The changes are indicated by designations from $\mp$ to 3+; or, in case of extreme changes 4+. At the end of the test, X-ray examination is again made of the left tibia under the same conditions.

Autopsy. The animals are killed with illuminating gas and autopsy is performed. This examination has aimed at the following points. (1) Whether the costochondral junctions are swollen, whether there are any so-called costal fractures, and whether there is a "Harrison's groove." (2) Whether the epiphyseal line of the proximal end of the left tibia is wider than normal. The normal width is taken as $\frac{1}{4}$ mm. or less [Schulz, 1929]. The greatest width of this epiphyseal line in rachitic rats has been 1–1$\frac{1}{4}$ mm. (3) Whether the bone is soft to the knife. No great importance has been attached to this point, however, on account of the subjective character of its estimation. The degree of the changes revealed by autopsy has been designated by values ranging from $\div$ to 3+, in particularly severe degree of rickets 4+.

Line test. The line test of Shipley has been carried out in the curative experiments. After the upper end of the left tibia has been divided by sagittal section, one of the cut surfaces is immersed in a 1 % silver nitrate solution, and in this solution it is irradiated by sunlight or by a mercury quartz lamp (Alpine Sun, Hanau); with the last source of radiation usually for 40 seconds. Then the cut surface is examined under a lens with 6 × magnification. Under the given experimental conditions the results obtained with this method have been so inconstant as to make their quantitative estimation questionable. Still the relative values have been entered in the Tables, and their significance will be discussed later. In the estimation of these findings, as far as possible, I have followed the scheme of Bills, but only from $\div$ to 3+; in several instances where such an estimation has been impracticable, this is shown in the table by a question mark. In computing the average values these doubtful animals are not taken into account.

Table I. *Preventive method.*

In every case the experimental period has been 28 days. X-ray examination was made of every animal at the beginning of the experiment, when all the rats were found free of rickets. The X-ray findings recorded are the results of the X-ray examination at the conclusion of each test.

Litter	Rat No.	Addition	Initial weight g.	Gain in weight g.	X-ray examination	Autopsy	Bone analysis: Dry substance %	Bone analysis: Ash %
491	1832 ♀	0	69	37	4+	3+	48·8	19·8
Age of mother: 10½ months.	1833 ♂	0	76	68	3+	3+	45·8	19·3
3rd litter	1834 ♂	1 mg. cod-liver oil	72	60	3+	2½+	62·5	24·5
	1835 ♀	1 ,,	64	43	3+	2½+	52·8	25·1
	1836 ♀	3 ,,	71	44	÷	÷	58·4	30·9
574	2098 ♀	0·2 g. peanut oil	46	29	3+	3+	45·0	12·7
Age of mother: 10 months.	2099 ♂	1 mg. cod-liver oil	49	40	2½+	2½+	43·0	14·2
4th litter	2100 ♂	2 ,,	43	39	½+	2+	46·9	17·0
	2101 ♂	2 ,,	47	31	÷	1+	50·8	19·4
	2102 ♀	3 ,,	47	17	÷	÷	59·4	24·5
	2103 ♀	3 ,,	46	25	÷	÷	53·4	22·6
	2104 ♀	5 ,,	43	25	÷	÷	56·9	23·1
	2105 ♀	5 ,,	46	22	÷	÷	54·0	19·7
	2106 ♂	10 ,,	50	31	÷	÷	55·2	23·7
586	2167 ♀	0·2 g. peanut oil	59	37	3+	3+	45·8	19·2
Age of mother: 15 months.	2159 ♂	1 mg. cod-liver oil	56	32	2+	2+	48·4	21·5
7th litter	2161 ♂	1 ,,	61	33	1+	1½+	52·8	23·9
	2160 ♂	2 ,,	59	38	÷	÷	53·8	25·5
	2162 ♀	2 ,,	50	29	÷	÷	53·7	24·9
	2164 ♀	3 ,,	63	40	÷	÷	53·8	25·5
	2165 ♂	3 ,,	53	39	÷	÷	53·6	24·4
	2163 ♀	5 ,,	50	29	÷	÷	56·2	27·8
	2166 ♀	10 ,,	53	42	÷	÷	54·9	28·0
640	2373 ♂	0·2 g. peanut oil	39	32	3+	3+	43·8	15·2
Age of mother: 4 months.	2374 ♀	1 mg. cod-liver oil	42	25	1½+	1½+	45·0	19·8
1st litter	2375 ♀	2 ,,	42	35	½+	½+	51·1	22·3
	2376 ♀	2 ,,	38	34	÷	½+	52·2	23·1
	2377 ♀	3 ,,	38	34	÷	÷	53·0	24·5
	2378 ♀	3 ,,	38	32	÷	÷	54·0	25·0
	2379 ♂	5 ,,	46	36	÷	÷	55·1	26·6
	2380 ♂	10 ,,	38	36	÷	÷	53·4	21·8
641	2381 ♀	0·2 g. peanut oil	46	34	3+	3+	45·3	17·5
Age of mother: 8 months.	2382 ♂	1 mg. cod-liver oil	51	30	2+	2+	48·5	19·8
2nd litter	2383 ♀	1 ,,	48	33	2+	2+	47·8	20·4
	2384 ♀	2 ,,	47	27	÷	1+	51·4	22·9
	2385 ♂	3 ,,	46	33	÷	÷	52·4	22·0
	2386 ♂	5 ,,	48	38	÷	÷	53·4	25·7
	2387 ♀	10 ,,	50	34	÷	÷	54·0	23·7
661	2448 ♂	0·2 g. peanut oil	42	30	3+	3+	44·7	14·8
Age of mother: 9 months.	2447 ♀	1 mg. cod-liver oil	36	26	2½+	2½+	49·0	19·9
3rd litter	2449 ♀	5 ,,	35	27	÷	÷	55·0	24·8
	2451 ♂	5 ,,	39	33	÷	÷	54·7	24·6
	2450 ♀	10 ,,	36	20	÷	÷	54·4	24·7
	2452 ♂	10 ,,	39	30	÷	÷	54·4	23·5
682	2546 ♂	0·2 g. peanut oil	30	28	4+	4+	40·0	12·9
Age of mother: 4 months.	2540 ♀	2 mg. cod-liver oil	32	33	÷	÷	55·2	23·4
1st litter	2541 ♀	5 ,,	29	32	÷	÷	54·7	24·8
	2542 ♀	10 ,,	32	42	÷	÷	52·1	24·5
	2543 ♀	10 ,,	30	34	÷	÷	54·1	23·0
	2544 ♀	20 ,,	30	43	÷	÷	54·1	25·6
	2545 ♀	20 ,,	29	39	÷	÷	52·4	23·9
689	2563 ♂	0·2 g. peanut oil	38	44	4+	4+	38·9	13·2
Age of mother: 7½ months.	2564 ♂	2 mg. cod-liver oil	37	37	3+	3+	44·6	17·8
3rd litter	2565 ♂	5 ,,	42	49	÷	÷	51·6	24·2
	2566 ♂	10 ,,	41	49	÷	÷	50·0	21·7
	2567 ♀	20 ,,	36	39	÷	÷	50·5	24·2
	2568 ♂	20 ,,	42	38	÷	÷	52·3	23·9
	2569 ♂	40 ,,	39	46	÷	÷	51·7	25·0
	2570 ♂	40 ,,	40	55	÷	÷	51·6	23·8

Table II. *Curative method.*

The fore-period of every litter has been 28 days, except litter 533 (26 days) and litter 600 (31 days). The after-period of every litter has been 7 days. R I means the X-ray examination at the beginning of the after-period, R II at its conclusion.

Litter	Rat No.	Addition	Initial weight g.	Weight increase: Fore-period g.	Weight increase: After-period g	R I	R II	Autopsy	Line test	Dry substance %	Ash %
524	1945 ♂	0	49	35	6	3+	3+	3+	÷	46·8	16·4
Age of mother:	1946 ♂	0	48	37	11	3+	3+	3+	÷	48·8	17·4
6½ months.	1947 ♂	5 mg. cod-liver oil	46	38	12	3+	2½+	3+	2+	46·5	17·7
3rd litter	1948 ♂	5 ,,	49	43	6	3+	2+	2+	3+	46·8	17·2
	1949 ♂	10 ,,	50	41	7	3+	2+	2+	3+	47·4	18·1
	1950 ♂	10 ,,	53	41	13	3+	2+	2+	3+	45·5	14·4
	1951 ♀	20 ,,	51	31	11	3+	½+	1+	3+	51·7	22·0
	1952 ♂	20 ,,	51	36	6	2+	½+	½+	3+	50·5	20·5
533	1962 ♀	0	62	39	÷2	2+	3+	3+	?	55·3	23·8
Age of mother:	1963 ♂	0	69	42	3	2+	3+	3+	÷	50·5	18·0
14 months.	1966 ♀	20 mg. cod-liver oil	59	38	2	3+	2+	1+	3+	53·2	21·8
6th litter	1965 ♂	20 ,,	63	46	6	2½+	½+	2+	?	52·6	22·9
	1964 ♂	40 ,,	62	43	÷4	3+	÷	½+	?	52·8	19·9
	1967 ♀	40 ,,	54	38	÷2	2+	÷	÷	3+	56·2	24·0
542	1991 ♂	0	58	45	6	3+	3+	3+	÷	45·1	13·3
Age of mother:	1992 ♀	0	51	42	5	2½+	2½+	2½+	?	49·8	20·4
7½ months.	1993 ♀	0	52	34	2	2+	2+	3+	÷	51·3	20·4
3rd litter	1994 ♂	10 mg. cod-liver oil	56	48	8	2+	1+	2½+	1+	52·0	19·4
	1995 ♂	20 ,,	54	45	4	3+	1½+	2+	2+	49·7	19·7
	1996 ♂	40 ,,	55	42	0	3+	1½+	2+	1+	48·8	17·7
	1997 ♂	40 ,,	52	42	7	3+	÷	1½+	3+	50·8	17·1
555	2052 ♀	0	50	25	5	3+	3+	3+	÷	49·8	17·7
Age of mother:	2053 ♂	10 mg. cod-liver oil	52	40	3	3+	1+	2+	2+	48·6	18·0
16 months.	2054 ♂	10 ,,	50	37	3	3+	½+	1+	½+	53·5	18·7
7th litter	2055 ♂	20 ,,	52	38	1	3+	½+	2+	2+	50·7	16·2
	2056 ♀	20 ,,	48	36	1	3+	1½+	2+	1+	50·9	21·3
	2057 ♂	40 ,,	51	36	2	3+	2+	1+	1+	49·8	19·1
566	2094 ♂	0·2 g. peanut oil	50	24	9	3+	3+	3+	÷	46·3	15·0
Age of mother:	2092 ♂	10 mg. cod-liver oil	50	32	5	3+	2+	2+	3+	49·1	20·0
8 months.	2093 ♀	10 ,,	45	33	3	3+	2+	3+	2+	45·9	18·7
4th litter	2091 ♂	20 ,,	47	37	6	3+	2+	2+	3+	44·3	17·8
	2095 ♀	20 ,,	47	30	3	3+	1+	1½+	3+	49·3	19·5
	2096 ♂	40 ,,	49	42	6	3+	2+	2½+	2+	42·8	16·2
	2097 ♂	40 ,,	49	39	3	3+	1½+	2½+	?	45·8	16·0
600	2230 ♂	0·2 g. peanut oil	50	45	8	3+	3+	3+	÷	43·3	15·0
Age of mother:	2236 ♀	10 mg. cod-liver oil	46	44	8	3+	2½+	3+	?	43·6	17·8
6½ months.	2235 ♂	10 ,,	48	46	8	3+	1+	1½+	?	46·8	19·6
2nd litter	2234 ♀	20 ,,	42	41	8	3+	1½+	½+	3+	50·2	23·6
	2233 ♀	20 ,,	52	41	10	3+	1½+	1+	3+	47·7	20·7
	2232 ♀	40 ,,	48	37	10	3+	½+	1+	3+	51·5	23·4
	2231 ♀	40 ,,	46	38	11	3+	÷	÷	3+	50·3	24·7
617	2282 ♂	0·2 g. peanut oil	43	27	3	2½+	2½+	3+	÷	43·9	15·2
Age of mother:	2283 ♀	10 mg. cod-liver oil	42	31	2	2½+	½+	1+	1+	46·1	18·5
11 months.	2284 ♀	10 ,,	42	28	4	3+	1½+	2+	1+	45·4	18·1
4th litter	2285 ♀	20 ,,	47	30	0	3+	2+	3+	2+	42·0	15·2
	2286 ♂	20 ,,	48	37	1	3+	1+	2+	3+	44·4	17·0
	2287 ♂	40 ,,	46	30	4	3+	÷	1+	2+	45·7	18·1
	2288 ♂	40 ,,	48	25	6	3+	1+	3+	2+	43·9	15·8

Analysis of the bones. At conclusion of the autopsy, the right tibia is dissected out rapidly. The greatest care has been employed in every instance to prepare the bone in the same way. The periosteum, menisci and ligaments are removed. The fibula is always cut off in the same place. The weighing of the bone is done as quickly as possible and the bone is then placed in an electrical drying-oven, where it is left for 72 hours at 100°. Such an intensive drying process is required, in order to ascertain the dry substance content of the bone. After 72 hours, the bones are constant in weight.

The incineration is done in a muffle-oven heated by 4 Méker burners. The temperature corresponds with that of light red heat. In some experiments with the purest $CaCO_3$ from Kahlbaum, it was ascertained that this temperature was sufficient to calcine this substance into CaO in less than $\frac{1}{4}$ hour. The bone is placed in the oven in a small porcelain crucible and calcined for $\frac{1}{4}$ hour. It is then taken out of the oven, cooled and moistened with 2 drops of concentrated nitric acid, after which it is again heated in the oven to a red heat for 10 minutes. In every instance this procedure has yielded a firm snow-white ash which does not lose in weight on further heating. After cooling in the desiccator, the final weight is recorded. The results of these examinations are given in detail in Tables I and II and are summarised in Tables III and IV, arranged according to the nature of the addition to the diet. Thus, each group comprises all the rats on the respective addition, giving the limits of variation of the dry substance percentage of the bones, ash percentage, X-ray findings, autopsy findings, and line test. The tables give further the average values obtained in these examinations, but under the last three headings the averages are entered in parentheses, as, I think, no great significance is attributable to these averages.

Table III. *Results obtained by the preventive method (Table I) arranged according to dose of addition.*

V means Extent of variation. A means Average.

Addition	Number of animals	Dry substance of bone %	Ash of fresh bone %	X-ray examination	Autopsy
Peanut oil or no addition	9	V: 38·9/48·8 A: 44·3	V: 12·7/19·8 A: 16·1	V: 3+/4+ (A: 3·3+)	V: 3+/4+ (A: 3·2+)
1 mg. cod-liver oil	9	V: 43·0/62·5 A: 49·5	V: 14·2/25·1 A: 20·6	V: 1+/3+ (A: 2·3+)	V: 1½+/3+ (A: 2·2+)
2 ,,	9	V: 46·9/55·2 A: 51·9	V: 17·0/25·5 A: 22·3	V: ÷/½+ (A: 0·1+)	V: ÷/2+ (A: 0·6+)
3 ,,	8	V: 52·4/59·4 A: 54·8	V: 22·0/30·9 A: 24·9	V: 0 (A: ÷)	V: 0 (A: ÷)
5 ,,	9	V: 51·6/56·9 A: 54·7	V: 19·7/27·8 A: 24·6	V: 0 (A: ÷)	V: 0 (A: ÷)
10 ,,	9	V: 50·0/57·4 A: 54·0	V: 21·7/28·0 A: 23·8	V: 0 (A: ÷)	V: 0 (A: ÷)
20 ,,	4	V: 50·5/54·1 A: 52·3	V: 23·9/25·6 A: 24·4	V: 0 (A: ÷)	V: 0 (A: ÷)
40 ,,	2	V: 51·6/51·7 A: 51·7	V: 23·8/25·0 A: 24·4	V: 0 (A: ÷)	V: 0 (A: ÷)

Table III shows a gradual rise of the averages with increase of the dose up to 3 mg. cod-liver oil, which is thus the preventive dose. The X-ray and the autopsy findings run very nearly parallel, falling off in + values up to the 3 mg. dose, at which both the X-ray examination and the autopsy give invariably negative results. From percentage of dry substance and of ash, X-ray and autopsy findings, it is consistently evident that cod-liver oil in doses above 3 mg. has had no additional effect. If, with this information in

mind, one turns back to Table I and surveys the material, litter by litter, it becomes strikingly obvious that practically every one of the litters that had a suitable dose of cod-liver oil allows of the same conclusion with regard to the size of the preventive dose. This applies in particular to the X-ray and the autopsy findings. This point is illustrated especially by the litters 574, 640, 661, 682 and 689, whereas litters 586 and 641 leave some doubt whether 3 mg. cod-liver oil has really produced the greatest possible effect on the dry substance and ash content of the bone. In these two litters, however, the X-ray and autopsy findings are in keeping with the findings in the other litters.

Table IV. *Results obtained by the curative method (Table II) arranged according to dose of addition.*

V means Extent of variation. A means Average.

Addition	Number of animals	Dry substance of bone %	Ash of fresh bone %	X-ray examination	Autopsy	Line test
Peanut oil or no addition	11	V: 43·3/55·3 A: 48·3	V: 13·3/23·8 A: 17·5	V: 2½+/3+ (A: 2·8+)	V: 2½+/3+ (A: 3·0+)	V: 0 (A: ÷)
5 mg. cod-liver oil	2	V: 46·5/46·8 A: 46·7	V: 17·2/17·7 A: 17·5	V: 2+/2½+ (A: 2·3+)	V: 2+/3+ (A: 2·5+)	V: 2+/3+ (A: 2·5+)
10 mg. cod-liver oil	11	V: 43·6/53·5 A: 47·6	V: 14·4/20·0 A: 18·3	V: ½+/2+ (A: 1·5+)	V: 1+/3+ (A: 2·0+)	V: ½+/3+ (A: 1·8+)
20 mg. cod-liver oil	13	V: 42·0/53·2 A: 49·0	V: 15·2/23·6 A: 21·4	V: ½+/2+ (A: 1·2+)	V: ½+/3+ (A: 1·6+)	V: 1+/3+ (A: 2·6+)
40 mg. cod-liver oil	11	V: 42·8/56·2 A: 48·9	V: 15·8/24·7 A: 19·3	V: ÷/2+ (A: 0·8+)	V: ÷/3+ (A: 1·4+)	V: 1+/3+ (A: 2·2+)

Table IV tells a rather different story. From the averages it will be noticed that the curative dose is about 20 mg. cod-liver oil. The averages of the percentage dry substance and ash show that the outcome of the test falls considerably short of complete recovery, these averages corresponding approximately with the results obtained with the 1 mg. dose in the preventive method. Besides, the difference of the averages is not so distinct by far as that in Table I, and the significance of the average values loses in weight on account of the wide range of the variations; moreover, the limits of variation for the different doses overlap to such an extent that they do not by themselves give any distinction between large and small doses. This is decidedly in contrast with the findings under the preventive method. With the preventive method the lower level of variation shows a gradual rise till the preventive dose is reached, after which this limit does not change to any significant degree.

In other words an attempt to estimate the curative dose by considering a single litter would be impossible; it would be necessary to work with the averages for several litters.

The X-ray and autopsy findings leave a similar impression. The average values of these examinations convey in all essentials the same impression as do the bone analyses; yet it should be pointed out that in these examinations

the lower limit of variation (corresponding to the highest degree of recovery) gives more exact information than does the corresponding limit in the bone analyses. Judging from this lower limit, 40 mg. cod-liver oil would give a greater effect than 20 mg., but I do not think that one may draw any conclusions as to this point from the relatively few animals, especially when the upper limit of variation comes as high as it does in these tests.

Of the line test, really, there is only this to be said, that it turned out negative in the negative control animals and positive in all the cases where cod-liver oil was given, leaving out those cases where the estimation was difficult on account of irregularities. The extent of variation and the average values do not allow of any conclusions.

Discussion.

It seems to me that these experiments suggest that in estimation of vitamin D the preventive method is preferable to the curative method. The experimental period is shorter, and there is less variation in the results from animals on the same addition. The latter point means that the estimation may be carried out with a smaller number of animals. In their employment, upon the whole, the preventive method is the more simple of the two. Here the point is to ascertain whether the test animal has rickets or not—which is comparatively easy with the criteria employed in this investigation. In the curative method the degree of rickets is the important point; and, as is evident from these experiments, this is not easy to estimate. One difficulty is involved in the relatively wide variation of the results in test animals on the same addition; in the bone analyses one may be somewhat justified in judging by an average value. The other difficulty arises if one wants an average estimate on the basis of the X-ray and autopsy findings together with the line test. In relying on an average of one's arbitrary gradations, it is prerequisite that these gradations express the degree of rickets in a certain regular progression—a presupposition that is naturally uncertain. The less arithmetic employed in these biological tests, the safer is the method.

If the question is raised whether the preventive method requires a comparison of the bone analyses, the X-ray examinations and the autopsy findings, in order to settle the presence or absence of rickets, a review of Table I will show that the X-ray examination and the autopsy have separately given the same result as the bone analysis.

Summary.

1. A precise description is given of the technique employed in the preventive and the curative methods for estimation of vitamin D. The results are judged by bone analysis, X-ray examination and autopsy.

2. Parallel series of experiments are made by the preventive and curative methods; the same cod-liver oil is used in both series.

3. A comparison of the results shows that the preventive method is superior to the curative, especially because the animals behave more alike in tests with the first method, so that each estimation can be carried out with a smaller number of animals.

4. A review of the records shows that the bone analyses do not give other or more uniform results than are obtained by X-ray examination and autopsy.

5. On the basis of these findings together with some general considerations, the preventive method is recommended as the standard method for estimation of vitamin D. X-ray examination or autopsy gives sufficient information for judging the results of the test.

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CXC. THE BLOOD-SUGAR LEVEL IN VITAMIN B_1 DEFICIENCY.

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THERE is still some uncertainty about the reducing value of blood in vitamin B deficiency. The recent contributions on this subject include that of Drummond and Marrian [1926], who found a rise in the blood-sugar of rats fed on a diet completely deficient in B-vitamins, and that of Sure and Smith [1929], who seemed to have provided an explanation when they reported that the rise was due to non-sugar reducing substances in the blood of vitamin B_1-deficient rats. Drummond and Marrian had used the original Hagedorn-Jensen method; Sure and Smith applied Folin's ferricyanide reduction method [1928] to the filtrates obtained by using the Folin-Wu tungstate precipitation [1919] and the Somogyi zinc hydroxide precipitation [1928]. In view of further recent work on the nature of the reducing substances of blood, it seemed desirable to re-investigate the problem, and the original idea was to ascertain what non-sugar reducing substance was responsible for the apparent rise in sugar.

As a preliminary to employing the methods at present regarded as orthodox [Cori, 1931] for arriving at "true sugar" and non-sugar reducing values, series of human bloods and of pigeons' bloods were analysed to compare the Somogyi and the Hagedorn-Jensen zinc filtrates. The Hagedorn-Jensen ferricyanide reduction was used throughout; the filtrates used were the Folin-Wu for total reduction, the Somogyi zinc filtrate, and, in pigeons, the unlaked filtrate of Herbert and Bourne [1930, 1] for "true sugar" values.

It has been shown that for normal and pathological human bloods [Herbert and Bourne, 1930, 2], the non-sugar reducing fraction runs parallel with the cell volume, as the greater part of this fraction is contained in the corpuscles. Accordingly, the cell volume was determined by half-an-hour's centrifuging in haematocrit tubes at 1500 revolutions. Sure and Smith's results seemed to indicate that there would be a very large increase in the cell volume or that there must be some entirely new reducing substance present in bloods from vitamin B_1-deficient animals.

In the following series, the bloods both of pigeons and of rats during normal and vitamin B_1-deficient periods, have been studied. Pigeons were studied in the first instance, partly because they could better spare the blood needed for

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repeated bleedings and for duplicate estimations, partly because they have a higher figure than rats both for sugar and for non-sugar reducing substances, as shown by Gulland and Peters [1930]; hence the risk of experimental error of any magnitude, with the micro-methods which must be used, would be lessened.

Experimental.

Comparison of Hagedorn-Jensen with Somogyi zinc filtrates from human blood.

It may seem unnecessary to produce further evidence that the Hagedorn-Jensen filtrate does not give absolutely true sugar values when several workers have already confirmed Somogyi's results [Herbert and Bourne, 1930, 1; Ege and Roche, 1930]; but as the Hagedorn-Jensen method of precipitation is still widely used, and as, for example, Gulland and Peters [1930] state that "zinc filtrates" (unqualified) "do not contain aliphatic —SS— and —SH— compounds," and that "zinc filtrates are the most reliable for estimating reducing substances in avian blood," it seemed advisable to go over this ground once more in order to see whether there were any significant differences between the two methods of zinc hydroxide precipitation. Somogyi claims that adjusting the reaction to neutrality or slight alkalinity not only precipitates the proteins completely, but also removes the non-sugar reducing substances.

In a recent communication, Kramer and Steiner [1931] have raised an objection to the use of cotton-wool plugs for filtering the coagulated proteins on the ground that they retained some of the sugar. Comparing this filtrate with that of Somogyi they obtained values of 20–30 mg. higher for human blood with the former than with the latter; Somogyi's [1930] comparison between these two methods similarly gave differences of 20–30 mg. On the other hand, Herbert and Bourne found values which were only 1–10 mg. higher; Ege and Roche similarly obtained only a 5–15 mg. difference. Ferricyanide reduction was used in all these instances. The discrepancies were explained by Kramer and Steiner by the inadequate washing of the cotton-wool plugs. In a private communication Herbert and Bourne state that they used, not cotton-wool, but 4 cm. filter-papers, and that a third washing did not alter their result. In order to clear up this point, the following procedure was adopted, using a micro-technique and ferricyanide reduction.

1. Somogyi's No. 1 procedure, *i.e.* using 1·8 % $ZnSO_4, 7H_2O$ and 0·1 *N* NaOH. The solutions were adjusted and precipitation effected as specified by Somogyi.

2. It will be observed that the actual amount of zinc sulphate added is four-fifths of that used in the Hagedorn-Jensen filtrate. (It may be remarked in passing that Hagedorn and Jensen do not state specifically in their original paper that it is the $ZnSO_4, 7H_2O$ which is to be used, but it is apparently the common practice to use the salt containing water of crystallisation.) Therefore, in order to make the two methods comparable in all respects except in the amount of zinc sulphate added, the Hagedorn-Jensen filtrate was prepared

according to the following directions. To 0·2 cc. blood, laked in 5·5 cc. water add 1·25 cc. of 1·8 % $ZnSO_4, 7H_2O$ and 1·0 cc. of 0·1 *N* NaOH. Place in boiling water-bath for 3 minutes. Filter through filter-paper (starch-free). Take 5 cc. of the filtrate. The procedure was the same, therefore, for the Hagedorn-Jensen as for the Somogyi technique, except that the amount of zinc sulphate was 1·25 times greater in the former than in the latter, and that there had been no application of heat in the latter. The following points were attended to: the amount of fluid in the boiling-tubes was made up to 14 cc.; the tubes were covered during and after reduction to minimise re-oxidation; the ferricyanide was twice recrystallised from a "recrystallised" sample. Care was taken to see that the blood was adequately mixed, to ensure even distribution of the corpuscles.

The figures obtained for human blood are given in Table I. It will be seen that the differences range from 5 to 15 mg. with an average of 8, which compares very closely with those recorded by Herbert and Bourne and by Ege and Roche.

Table I. *Comparison between Somogyi and Hagedorn-Jensen filtrates. Human blood.*

	Sugar value (mg./100 cc.)		
Sample	Somogyi filtrate	Hagedorn-Jensen filtrate	Difference
1	90	105	15
2	116	123	7
3	105	112	7
4	102	109	7
5	90	99	9
6	64	69	5
7	73	86	13
8	88	93	5
9	70	80	10
10	80	86	6
		Average	8

The cotton-wool plugs accordingly do not seem to explain the discrepancy, as suggested by Kramer and Steiner. The latter state that they used 0·30 to 0·35 cc. *N*/10 NaOH to neutralise their filtrate. As it seemed possible that they might perhaps have made their zinc sulphate solution to contain 0·45 % of $ZnSO_4$ instead of 0·45 % $ZnSO_4, 7H_2O$, a few human bloods were tested using the additional zinc sulphate, neutralising and not neutralising the filtrate, and again taking an aliquot part of the filtrate. The effect of the extra zinc sulphate was to increase the difference in reduction between the Somogyi and the second filtrate only if the latter were neutralised. Apparently, not neutralising the filtrate diminished the efficacy of the ferricyanide reagent, with the result that the figures were much the same as the ordinary Hagedorn-Jensen figures.

Herbert and Bourne have shown that the p_H of the Hagedorn-Jensen filtrate is 6·8, while that of the Somogyi filtrate is 7·4. In this investigation, the p_H was similarly determined on a few samples, using the method of Dale and Evans [1920]. The average values thus obtained were 6·85 and 7·2 re-

spectively. It would seem, therefore, that the critical p_H is the neutral point, as Somogyi claims.

Reducing values for pigeons' blood.

Whereas the evidence put forward by several different workers is that the two zinc filtrates give slightly different results for human blood, the difference is more marked when they are applied to avian blood, with its greater content of non-sugar reducing substances. It was stated by Gulland and Peters that 70 mg. in terms of glucose was the average for the non-sugar reducing substances of pigeons' blood. They, however, used the Hagedorn-Jensen filtrate to give total reduction, and blood which has been allowed to undergo slow spontaneous glycolysis to obtain their residual reduction. The Hagedorn-Jensen filtrate, as Table II shows, does not allow through more than a part of

Table II. *Comparison between Hagedorn-Jensen*[1] *and Somogyi filtrates. Pigeons' blood.*

	(mg./100 cc.)			
No.	"Total sugar" (Folin-Wu filtrate)	Somogyi filtrate	Hagedorn-Jensen filtrate	Difference
1	256	192	208	16
2	256	198	230	32
3	227	176	207	31
4	254	198	224	26
5	315	259	291	32
6	334	279	300	21
7	230	163	190	27
8	227	176	207	31

the non-sugars, and it is possible that blood after slow glycolysis has not lost all its glucose. Moreover, in the absence of glucose, as Herbert, Bourne and Groen [1930] have shown, glutathione has a rather higher glucose equivalent. It is suggested, therefore, that the figure of 70 mg. was obtained by a compensation of errors.

Blood was obtained by puncturing the wing vein, the bird being kept quiet by putting its head under the opposite wing. The blood was rapidly mixed with a minimal quantity of oxalate. 0·1 cc. blood was used for an estimation in each case.

Normal birds were fed with poultry feed. The deficient diet offered was polished rice and Kon and Drummond's diet [1927]. When the birds refused to eat the deficient diet, they were forcibly fed with the latter. About 15 g. were placed in the crop each day; this was insufficient to maintain their weights, but ensured that they were not suffering from starvation.

It will be seen (Tables III and IV) that the average non-sugar reducing value for pigeons' blood by these methods is about 60 mg. in terms of glucose. The cell volume averages 49 %. For human blood, the corresponding figures are 20–30 mg. and 41 % respectively [Herbert and Bourne, 1930, 2]. The

[1] Using an aliquot part of the filtrate.

Table III. *Normal pigeons fasting 24 hours.*

Bird	Date	Reduction value expressed as mg. glucose/100 cc. blood: "Total sugar" Folin-Wu filtrate	"True sugar": Somogyi filtrate	"True sugar": Isotonic filtrate	Non-sugar reducing substances	Cell volume %
"Squeakers"						
734	8. vii. 31	—	130	—	—	—
734	17. vii. 31	206	133	130	73	51
736	8. vii. 31	243	182	—	61	—
736	17. vii. 31	264	202	206	62	—
742	8. vii. 31	206	150	—	56	46
742	17. vii. 31	184	130	133	54	—
478	8. vii. 31	203	136	139	67	51
478	17. vii. 31	224	154	—	70	52
731	8. vii. 31	205	150	150	55	46
731	17. vii. 31	221	154	—	67	—
	Average	217	152	—	63	49
Adult birds						
p.s.r.	19. v. 31	249	181	178	68	49
1707	19. v. 31	243	179	—	64	—
9754	19. v. 31	245	179	181	66	50
1478	19. v. 31	252	194	—	58	45
p.p.r.	20. v. 31	238	179	174	59	50
9738	20. v. 31	229	167	167	62	51
9731	22. v. 31	254	198	198	56	—
9743	22. v. 31	237	176	176	61	47
	Average	243	182	—	62	49

Table IV. *Normal pigeons feeding.*

Bird	Date	Reduction value expressed as mg. glucose/100 cc. blood: "Total sugar" Folin-Wu filtrate	"True sugar": Somogyi filtrate	"True sugar": Isotonic filtrate	Non-sugar reducing substances	Cell volume %
"Squeakers"						
736	2. vii. 31	246	195	—	51	—
734	2. vii. 31	242	174	—	68	55
731	2. vii. 31	253	184	184	69	47
742	2. vii. 31	235	165	165	70	49
478	2. vii. 31	216	162	—	54	51
	Average	238	176	—	62	50
Adult birds						
9742	25. iv. 31	249	195	192	54	43
375	27. iii. 31	283	229	—	54	—
375	25. iv. 31	293	228	222	65	54
9736	31. iii. 31	246	187	187	59	—
p.p.	31. iii. 31	278	—	—	—	—
9735	7. iv. 31	258	206	—	52	—
1478	7. iv. 31	248	203	200	45	—
m.f.	8. iv. 31	268	208	202	60	—
f.w.	8. iv. 31	246	187	—	59	—
9731	8. iv. 31	253	195	—	58	46
9743	8. iv. 31	264	197	200	67	45
1707	9. iv. 31	254	206	—	48	—
9738	10. iv. 31	277	216	—	61	—
p.w.	17. iv. 31	—	212	213	—	—
p.s.r.	26. iii. 31	256	192	—	64	—
p.w.	26. iii. 31	256	198	—	58	47
	Average	262	204	—	58	47

non-sugar reducing content is consequently higher per unit volume of corpuscles in birds' blood than in human blood.

The Hagedorn-Jensen filtrate (Table II) gives values 16–32 mg. higher than the Somogyi filtrate, but not as high as the Folin-Wu filtrate. When these filtrates were tested by the nitroprusside reaction, the Folin-Wu gave a markedly positive colour, the isotonic and the Somogyi were negative, while the Hagedorn-Jensen gave a colour about half as intense as the Folin-Wu. This lends support to the view that the substance mainly concerned in producing non-sugar reduction is glutathione or other sulphydryl-containing substances.

The "true sugar" averages 152 mg. for young birds fasting 24 hours, 182 mg. for adult birds fasting 24 hours; 176 mg. for young birds feeding, 204 mg. for adult birds feeding. The range of figures is a wide one, namely from 130 mg. to 198 mg. for fasting birds, from 162 mg. to 230 mg. for feeding birds. This wide range is probably to be explained by the varying degrees of excitement that the manipulations produce. From the small group of young birds studied, the impression was gathered that the smaller birds had lower amounts of circulating glucose, perhaps connected with their greater intensity of metabolism and more rapid utilisation of glycogen stores. Gulland and Peters deduced from their average of 135 ± 15 mg. glucose that "the true sugar value of birds' blood is not so widely divergent from that of mammals as the Hagedorn-Jensen estimations seem to show." With the methods used here, the figures are higher, in spite of the fact that their method of collecting the sample—by guillotining the bird—is likely to lead to a rise in blood-sugar. One is of the opinion, therefore, that their figures for true sugar are definitely low.

The two methods for true sugar were undertaken, firstly in order to see what effect the extra amount of uric acid contained in birds' blood had on the unlaked filtrate of Herbert and Bourne. Their filtrate is practically identical with that simultaneously proposed by Folin [1930]. It is unlikely that the uric acid would be confined to the corpuscles, and yet the figures show rather surprising agreement between the unlaked and the Somogyi filtrate. The figures given by Folin and Denis [1913] for uric acid in birds' blood are 4·8 mg.; those given by Scheunert and Pelchrzim [1923] are about 2 mg. The reducing value of uric acid, using ferricyanide reduction, is 53 % that of glucose [Hagedorn and Jensen, 1923, 2; Holden, 1926], so that the maximum difference due to uric acid would be 2·4 mg. in terms of glucose, even if it all diffused out of the corpuscle in the time allowed. The second reason for using the unlaked filtrate was to see whether the alleged rise in non-sugar reducing substances in vitamin B_1-deficiency were due to a constituent of the cells or of the plasma; if it were a non-diffusible substance present in the cells, then the isotonic filtrate would show a smaller value than the Somogyi filtrate. It was found, as later results indicate, that the two filtrates gave very similar results during the stage of vitamin B_1-deficiency in pigeons.

Table V. *Vitamin B_1-deficient pigeons during convulsions.*

Bird	Date	Reduction value expressed as mg. glucose/100 cc. blood: "Total sugar" Folin-Wu filtrate	"True sugar": Somogyi filtrate	"True sugar": Isotonic filtrate	Non-sugar reducing substances	Cell volume %
Adult birds						
p.s.r.	13. iv. 31	507	461	—	46	37
	14. iv. 31	392	336	336	66	35
9754	18. iv. 31	264	197	198	66	43
	19. iv. 31	314	—	258	56	46
	20. iv. 31	382	312	312	70	44
	21. iv. 31	333	—	—	—	—
p.p.	18. iv. 31	340	282	288	58	39
	19. iv. 31	370	314	325	56	37
	20. iv. 31	298	—	261	37	35
9738	22. iv. 31	298	235	228	63	—
f.w.	22. iv. 31	320	275	273	45	38
	23. iv. 31	480	438	419	42	—
	24. iv. 31	—	509	—	—	—
m.f.	23. iv. 31	344	296	296	48	—
	24. iv. 31	299	256	267	43	—
1478	27. iv. 31	406	330	—	76	—
	28. iv. 31	296	232	—	64	—
	29. iv. 31	283	243	245	40	35
9743	28. iv. 31	296	248	251	48	40
	29. iv. 31	259	221	230	38	37
1707	1. v. 31	—	237	243	—	40
	2. v. 31	279	229	235	50	—
9731	8. v. 31	242	197	—	45	32
p.w.	15. v. 31	374	317	306	57	44
9736	23. v. 31	338	253	—	85	48
p.s.	11. vi. 31	262	219	222	43	40
	12. vi. 31	355	296	—	59	—
	13. vi. 31	—	283	—	—	—
	14. vi. 31	315	232	—	83	42
9731	15. vi. 31	—	295	—	—	—
9734	15. vi. 31	340	275	—	65	—
9742	17. vi. 31	229	155	156	74	51
	17. vi. 31	248	190	—	58	—
	18. vi. 31	289	—	228	61	53
	18. vi. 31	412	350	—	62	52
	19. vi. 31	459	398	396	61	52
p.w.	18. vi. 31	515	434	440	75	41
	18. vi. 31	506	459	—	47	—
	19. vi. 31	367	314	—	53	45
9743	19. vi. 31	—	248	251	—	39
p.p.	20. vi. 31	278	—	224	54	48
	20. vi. 31	314	267	—	47	47
1478	20. vi. 31	294	249	—	45	44
9736	21. vi. 31	—	262	—	—	—
	Average	340	290	—	53	41
"Squeakers"						
478	30. vii. 31	334	—	279	55	42
	31. vii. 31	302	253	—	49	43
	1. viii. 31	302	256	—	46	40
	2. viii. 31	—	253	—	—	40
	3. viii. 31	—	267	—	—	—
731	31. viii. 31	534	499	—	—	44
	1. viii. 31	397	336	—	61	—
742	5. viii. 31	251	195	—	56	—
	6. viii. 31	235	165	—	70	60
	6. viii. 31	236	—	151	85	65
734	6. viii. 31	211	170	—	41	—
	7. viii. 31	—	224	—	—	—
	Average	311	272	—	59	49

Blood-sugar changes in pigeons during vitamin B_1-deficiency.

The changes that occurred in "true sugar" during vitamin B_1-deficiency can be seen in Tables V, VI, VII and Fig. 1. During the stage of convulsions, the "true sugar" went up—parallel with the "total sugar"—to an average value of 290 mg. for adult birds and 272 mg. for the group of young birds.

Table VI. *"True sugar" in blood of pigeons during pre-convulsive stage.*

Bird	Date	"True sugar" (Somogyi filtrate) mg./100 cc.	Bird	Date	"True sugar" (Somogyi filtrate) mg./100 cc.
734	4. viii. 31	144	9735	12. v. 31	163
742	4. viii. 31	190	375	12. v. 31	190
734	31. vii. 31	154	p.w.	11. v. 31	182
731	31. vii. 31	224	9731	7. v. 31	218
742	31. vii. 31	176	p.p.r.	16. iv. 31	179
1478	16. vi. 31	173	9738	16. iv. 31	172

Table VII. *"True sugar" in blood from heart of pigeons, dead from vitamin B_1 deficiency.*

No. 742, at death, following convulsions ... 151 mg.
„ 9744, „ (from ascites) ... ... 312 mg.

Blood from heart after death:

No.	478	... ...	90 mg.
„	731	... ...	not less than 256 mg.
„	9731	... ...	27 mg.
„	9734	... ...	275 mg.
„	p.s.r.	... ...	128 mg.
„	734	... ...	378 mg. (dead in position of head retraction)

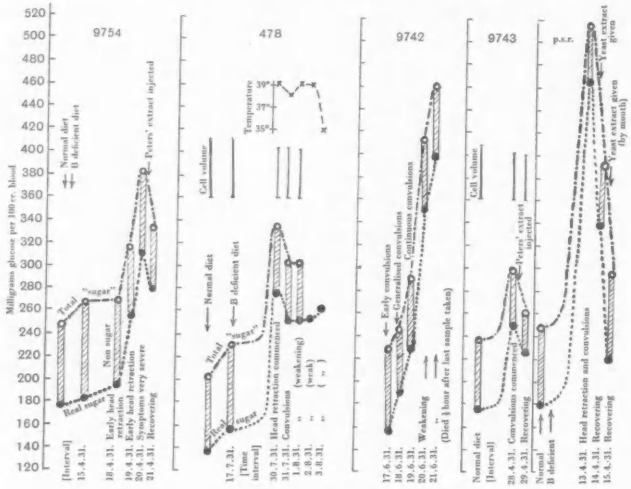

Fig. 1. Graphs of increased reduction in blood of pigeons during convulsions.

Occasionally the "true sugar" reached a figure of 500 mg. Of the twenty birds used, most of which were studied through two periods of deficiency, there was no exception to the finding that a given individual bird will show an increase over its fasting level for sugar, and usually over its feeding level also. The response varied considerably with different individual birds.

The non-sugar reducing substances suffered no appreciable alteration. Occasional high values were attributed to experimental error. The average value was 53 mg., in terms of glucose, for one group of animals, a rather lower figure than the normal average. In this group, furthermore, the average cell volume diminished slightly. Some birds showed a progressive anaemia—and the non-sugar reducing substances followed the cell volume downwards. This is consistent with the finding that the greater part of the non-sugar reducing substances resides in the corpuscles. Occasionally, on the other hand, a bird which developed ascites showed an increase both in cell-volume and in non-sugar reducing substances. Bird 742 is an extreme example of this, where the cell volume at the time of death was 65 %, and the non-sugar value was 85 mg. This was, however, exceptional. The bird had lost water not only into its tissues, but also by the rapid respiration, presumably due to hydropericardium, which it had been experiencing for several hours before death.

Occasionally a bird which did not develop convulsions had a high "true sugar" level, *e.g.* bird 9744 died during manipulations for taking the blood sample. It was found to have ascites, with fluid in the pericardial cavity. Its "true sugar" was at the high level of 342 mg. Undoubtedly an asphyxial element would enter into the production of hyperglycaemia in this case. Only twice was it possible to get blood from the heart immediately after death from convulsions. No. 742 whose temperature was 26° at death had a "true blood-sugar" of 150 mg.; No. 9742 died half-an-hour after the last sample containing 390 mg. had been taken. Even among the birds which had been dead for several hours before their heart-blood was collected, there were three with figures of 256, 275, and 378 mg. respectively. The lower figures of 90 and 27 mg. may have resulted from glycolysis, but may also have been similar findings to those of Drummond and Marrian who frequently found low values at the time of death.

On the whole, in this series, the incidence of hyperglycaemia has been associated with the convulsive stage. It was seldom that any rise occurred in the pre-convulsive stage (Table VI). Extraordinarily high figures were reached in some cases where convulsions were present. A sub-lethal dose of cyanide was accordingly administered to two pigeons to ascertain the effect of convulsions produced in this way. The first developed typical convulsions and gave an increase in "true sugar" from 131 to 259 mg. The second, dosed on two occasions, did not become convulsed, and showed a diminution from 139 to 128 mg. and from 160 to 149 mg. The hyperglycaemia in the first pigeon would no doubt come under the heading of an asphyxial rise in sugar [Macleod, 1926].

An attempt to prove that the raised blood-sugar was due to an increased output of adrenaline was unsuccessful.

Sugar values for rats' blood.

Since vitamin B_1-deficient pigeons had shown no rise in non-sugar reducing substances but were definitely hyperglycaemic during the convulsive stage, rats were now used as the experimental animals.

Normal values were first established and were found to lie, as Sure and Smith stated, within the values of 17–34, with an average of 29 mg. per 100 cc. of blood, in terms of glucose. In the first estimations, a number of (feeding) stock rats were used; they were killed with chloroform, and blood was taken from the heart. A few (feeding) vitamin B-deficient rats were similarly used. For the great majority of the figures, however, blood was taken from the lateral vein of the tail of animals fasting 6 hours or longer. From 0·2 to 0·3 cc. blood was collected with a pipette into a minimal quantity of oxalate.

The vitamin B_1-deficient animals were fed on a diet consisting of rice starch 75 %, caseinogen 20 %, salt mixture 5 %. A supplementary ration of 1 drop of cod-liver oil, and of autoclaved marmite (to supply vitamin B_2) was fed to all the rats; the controls received an additional ration of Peters's extract (vitamin B_1). The animals fasted for at least 6 hours before the sample was taken.

Normal values.

(*a*) "Total sugar"—usually below 120 and above 80 mg., with an average of 109 mg.

(*b*) "True sugar"—usually below 90 and above 50 mg., with an average of 76 mg.

(*c*) Cell volume, average value 44 %.

(*d*) Non-sugar reducing substances, average 29 mg. in terms of glucose.

The vitamin B-deficient animals were classified into those that had convulsions, those that did not develop convulsions, and those that were moribund when the sample was taken (Table VIII). The average cell volume for these groups was 44, 46, and 44 respectively. No consistent alteration occurred in the cell volume. The non-sugar reducing substance averaged 32, 28 and 27 mg. respectively, and in no case was it over 35 mg. Care was taken, when it was suspected that the "real sugar" might be low, to increase the quantity of blood taken for the Somogyi estimation from 0·1 to 0·15 cc. or more if it could be obtained. Duggan and Scott [1926] and Kramer and Steiner [1931] have thrown doubt on the accuracy of estimations of amounts below 25 mg. If that is the case, then a Somogyi estimation, since it is done on five-eighths of the filtrate, must be over 40 mg. to be accurate.

While, contrary to Sure and Smith's finding, no increase was found in the non-sugar reducing value of the blood of vitamin B-deficient rats, on the other hand, in agreement with Sure and Smith, no increase in the "true sugar" was

Table VIII. *Vitamin B-deficient rats, fasting 6 hours.*

No.	Date	"Total sugar"	"True sugar"	Non-sugar reducing substances	Cell volume
		(Convulsions.)			
124	9. vii. 31	71	41	30	44
49	9. vii. 31	123	91	32	43
50	9. vii. 31	63	28	35	—
121	9. vii. 31	96	68	28	—
941	12. vii. 31	—	83	—	—
48	14. vii. 31	111	77	34	—
119	18. vii. 31	101	72	29	45
125	20. vii. 31	112	79	33	—
	Average	97	67	32	44
		(No convulsions. Hunched.)			
43	29. vi. 31	75	53	22	—
40	2. vii. 31	98	75	23	—
122	2. vii. 31	117	100	17	42
117	11. vii. 31	143	118	25	44
126	5. viii. 31	91	74	17	—
128	7. viii. 31	110	78	32	48
125	7. viii. 31	103	74	29	48
126	7. viii. 31	99	64	35	48
43	7. viii. 31	112	83	29	42
46	7. viii. 31	100	70	30	—
50	10. viii. 31	96	—	—	45
49	10. viii. 31	120	96	24	41
129	12. viii. 31	90	64	26	—
133	12. viii. 31	86	54	32	47
132	12. viii. 31	83	54	29	46
131	12. viii. 31	96	72	24	—
130	12. viii. 31	102	68	34	—
132	19. viii. 31	82	56	26	—
51	20. viii. 31	101	72	29	—
39	18. vi. 31	106	74	32	46
40	18. vi. 31	102	70	32	45
	Average	101	73	28	46
		(Moribund.)			
52	29. vi. 31	120	90	30	42
42	2. viii. 31	130	101	29	—
123	10. vii. 31	67	38	29	—
122	10. vii. 31	—	7	—	—
118	10. vii. 31	40	15	25	46
119	20. vii. 31	25	3	22	—
	Average	77	42	27	44

observed. Not even during the stage of convulsions did the rats show a hyperglycaemia. Then began a process of elimination to account for the discrepancy between the findings in this series and the finding by Drummond and Marrian —that a "hyperglycaemia occurs in rats during the greater part of the time that they are on a vitamin B-deficient diet." Firstly, the Hagedorn-Jensen method was too little different from the Somogyi method to account for the discrepancy. Secondly, the diet given by Drummond and Marrian was deficient in B-complex; therefore a few rats deficient in vitamin B_2 only were used, to see whether this was the factor responsible, with the result that they also showed the same normal sugar values. Thirdly, as it was found that Drummond and Marrian had used blood taken from the heart immediately after rapidly

Table IX. *"True sugar" values for rats' blood, showing effect of method by which blood is collected.*

(Rats fasting overnight.) "True sugar" values (Somogyi filtrate).

	mg./100 cc. blood		
Normals No.	Blood from tail	Blood from heart, immediately after rapid death by dislocation of the neck	Rise produced by latter procedure
168	82	112	30
169	74	107	33
173	52	52	0
175	102	106	4
166	106	129	23
170	102	171	69
134	89	96	7
135	85	152	67
172	96	103	7
171	79	126	47
165	70	106	36
1	71	120	49
2	83	96	13
3	68	129	61
4	58	86	28
5	78	96	18
Vitamin B_1-deficient:			
132	56	79	28
125	79	118	39
51	62	79	17
133	48	82	34
130	40	49	9
49	81	139	58

killing the animals by dislocating the neck, a few vitamin B_1-deficient animals were treated in this way. Blood was first taken from the tail, the animals were rapidly killed and blood was taken immediately from the right side of the heart. These latter samples gave higher values than the tail-blood samples. In a series of normal rats treated in the same way, a similar rise was observed in the majority of the second samples. Sometimes there was no appreciable rise. The increase ranged from 0 to 69 mg. (Table IX). This means that it is possible to have something like a 100 % error if blood is collected by this method for sugar estimations. Apparently this fact has not been generally recognised hitherto. The rise occurs with extraordinary rapidity. The physiological explanation is either that of intense excitement before death, or direct stimulation of the spinal cord by the odontoid process of the second cervical vertebra as it penetrates the spinal cord; adrenaline secretion will occur and glycogen be mobilised. As evidence of adrenaline secretion, the blood clotted very rapidly.

No explanation, therefore, has been found for the definitely lower values for blood-sugar found by Drummond and Marrian to occur in the blood of normal rats as compared with vitamin B-deficient rats and at present it appears to be due to coincidence. It will be seen by reference to Table IX, that there is room for that coincidence to have occurred.

Discussion.

These results seem to explain why a hyperglycaemia in pigeons has not always been found. Funk and Schönborn [1914] and several other workers found a rise, but Kon and Drummond [1927] reported no difference in the sugar levels of the two groups of animals which they studied.

It appears to depend upon the stage at which the blood-sample is taken, the hyperglycaemia being definitely associated with the appearance of convulsions. As to the mechanism which produces the rise in blood-sugar, there is no satisfactory evidence. The most probable supposition is that a nervous stimulation occurs, producing adrenaline secretion and consequent mobilisation of glycogen. The absence of a detectable increase in blood-glucose in the rat during convulsions is probably to be explained by depletion of its carbohydrate stores. Kinnersley and Peters [1930] have recently reported finding a local production of lactic acid in the brain of vitamin B-deficient pigeons. If that lactic acid is a cause and not an effect of local stimulation, it may conceivably also be the cause of a *piqûre* type of effect giving rise to adrenaline secretion.

Finally, the author is not of the opinion that the finding of the hyperglycaemia throws any light on the essential nature of vitamin B_1-deficiency. It appears to be a subsidiary effect; the birds are definitely not well before they show the hyperglycaemia; the cause of failure to grow and to maintain health is not explained by it.

Summary.

1. A comparison between the Hagedorn-Jensen and the Somogyi methods of zinc hydroxide precipitation in human blood gave an average value of 8 mg. higher reduction with the former than with the latter.

2. A similar comparison on pigeons' blood gave values 16–32 mg. higher with the former than with the latter.

3. Using Folin-Wu, Somogyi and Herbert and Bourne filtrates, "total sugar," "true sugar," and non-sugar reducing values for fasting, adult pigeons' blood have averaged respectively 243, 182, 62 mg.

4. The isotonic filtrate of Herbert and Bourne gives practically the same values as the Somogyi filtrate for birds' blood.

5. There is a marked rise in "true sugar" during the convulsive stage in vitamin B_1-deficient pigeons.

6. No increase was found in the non-sugar reducing substances or cell volume of the blood of vitamin B_1-deficient pigeons or rats.

7. No increase was found in the "true sugar" of the blood of vitamin B_1-deficient rats.

8. There is usually a fall in blood-sugar in the final stages of vitamin B_1 deficiency in rats, but pigeons dying from convulsions or from hydropericardium are often hyperglycaemic.

9. When blood is obtained from the hearts of rats which have been rapidly killed by dislocation of the neck, it shows an increased sugar content in most

cases compared with circulating blood obtained immediately beforehand from the tail.

10. The hyperglycaemia in pigeons is thought to be the result of nervous stimulation—something of the nature of the hyperglycaemia of asphyxia, anaesthesia or diabetic *piqûre*. Its failure to occur in convulsive rats is ascribed to exhaustion of glycogen stores.

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CXCI. THE BIOLOGICAL DECOMPOSITION OF PLANT MATERIALS.

V. SOME FACTORS DETERMINING THE QUANTITY OF NITROGEN IMMOBILISED DURING DECOMPOSITION.

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The decomposition of plant materials in the soil or in compost heaps involves the assimilation of the carbohydrate constituents of the tissues and the conversion of available nitrogen to microbial protein by the organisms concerned. In nature the supply of nitrogen is often a limiting factor in determining the rate and extent of decomposition. Mature plant tissues frequently contain rather a low percentage of nitrogen, and to effect a complete and speedy rot, an additional quantity of available nitrogen has to be supplied. If too much is provided, losses occur, or if too little, the decomposition is incomplete unless an unusually long period of time is allowed. Hutchinson and Richards [1921] were the first to recognise the quantitative importance of nitrogen in decomposition, and they determined the amount necessary for the decomposition of a number of materials. For common straws, for example, they found it to be between 0·7 and 0·8 g. nitrogen per 100 g. original material. This figure they termed the "nitrogen factor." It may be defined as the additional inorganic nitrogen immobilised as organic nitrogen by 100 g. of any material in the process of decomposition. This quantitative relationship forms the basis of Richards and Hutchinson's patents [1924] for the production of "artificial farmyard manure" (Adco), from waste plant materials. The "nitrogen factor" gives the maximum amount of nitrogen which when added to decomposing material will be retained without loss.

Table I gives the "nitrogen factors" for a number of common materials, and indicates the range of variation found.

No basis exists for any prediction of the "nitrogen factor" and it has to be determined by trial in each case. The variations which are observed clearly depend on the differences in composition of the various materials and their relative availabilities to the micro-flora present. Accordingly investigations were undertaken to ascertain the nature of the relationship, if any, between

Table I. *"Nitrogen factors" of some waste plant materials.*

Plant material	"Nitrogen factor"	Loss of dry matter %
Willow peelings	1·33	35·2
Sugar cane trash	1·06	27·9
Oat straw	0·85	53·1
Rye straw	0·71	45·0
Rice straw	0·64	35·2
Esparto grass waste	0·52	25·7
Rushes (Scilly Isles)	0·50	21·9
Almond husks	0·44	55·0
Banana leaf	0·42	45·7
Almond skins	0·30	52·2
Flax shives	0·12	19·6

composition and "nitrogen factor." A number of widely different materials, some extracted suitably to remove known constituents, were employed, and rotted under optimum and parallel conditions.

Experimental.

The materials were in each case cut to a suitable size for rotting, about that of chaffed straw. Equal amounts of each, of known moisture content, were taken and moistened thoroughly by spraying. Available nitrogen in the form of ammonium carbonate was added to the material when bottled, together with an inoculum from soil in which organic matter was rotting. The moisture content was kept equal amongst the members of each series. In the case of certain extracted materials moistening was carried out with a mineral salt solution, since the process of extraction was likely to remove necessary ions. Washing in many cases had to be very prolonged to remove all traces of the extracting agents.

For the determination of the "nitrogen factor" the following figures were necessary.

1. Original nitrogen content—Kjeldahl.
2. Final nitrogen content—Kjeldahl.
3. Final ammonia content—distillation with MgO.
4. Loss of organic matter in decomposition.

To determine the composition of the materials employed, the following estimations were carried out as described by Norman [1929].

5. Total furfuraldehyde yield.
6. Cellulose (Cross and Bevan).
7. Furfuraldehyde from Cross and Bevan cellulose.
8. Carbon dioxide yield (uronic acid).

The composition and the "nitrogen factor" at various stages were determined for the materials enumerated in Table II.

Willow peelings were also rotted without the addition of nitrogen and this series will be designated D 2.

Table II. *Percentage composition of plant materials employed.*

Series No.	Plant material	C. and B. cellulose	Furfurald. from C. and B. cellulose	CO_2 yield	Total uronic anhydride	Furfurald. from uronic acids	Total furfurald. yield	Furfurald. from pentoses in hemicelluloses	Pentoses in hemicelluloses as anhydroxylose	Xylan associated with C. and B. cellulose	"Pure" cellulose	Lignin	Total N
A	Flax straw	53·40	6·49	1·69	6·76	1·13	12·04	4·42	6·9	10·1	43·3	30·5	0·51
B	Flax shives (retted)	53·57	6·88	1·29	5·16	0·86	12·56	4·82	7·5	10·7	42·9	30·9	0·21
C	Flax fibre	87·50	1·19	0·22	0·88	0·15	1·85	0·51	0·8	1·8	85·7	Nil	0·55
D	Willow peelings	31·16	1·80	2·69	10·76	1·79	7·37	3·78	5·9	2·9	28·3	36·5	1·58
E	Oat straw	48·92	6·61	1·36	5·44	0·91	15·95	7·43	11·5	10·25	38·7	18·5	0·30
F	Oat straw extract with hot water	50·99	6·97	1·56	6·24	1·04	16·59	8·58	13·3	10·8	40·2	—	0·27
G	Oat straw extract with 4 % NaOH	84·07	7·86	0·26	1·04	0·17	9·01	0·98	1·5	12·8	71·3	11·1	0·12
H	Oat straw extract with 2 % HCl	61·98	1·53	0·61	2·44	0·40	2·57	0·64	1·05	2·4	59·6	25·6	0·34
I	Oat straw extract with 4 % alc. NaOH	65·60	8·70	0·80	3·20	0·53	14·08	4·85	7·5	13·5	52·1	8·2	0·11
J	Young barley straw extract with 4 % NaOH	89·10	2·66	0·31	1·24	0·21	3·55	0·68	1·1	4·1	85·0	—	Nil
K	Esparto grass waste	48·07	5·91	—	—	—	12·66	6·8 (approx.)	10·6	9·2	38·9	26·7	0·95
L	Esparto grass waste extract with 4 % alc. NaOH	55·37	9·45	—	—	—	14·65	5·2 (approx.)	8·1	14·6	40·7	12·3	0·23

The composition of these materials is given in Table II and the "nitrogen factor" determinations in Table III. In addition to the "nitrogen factor," the "nitrogen equivalent" has been calculated. This represents the amount of additional nitrogen immobilised per 100 g. of organic matter lost. The use of this figure as an aggregate measure of efficiency of the organisms has been described in Part IV of this series [Norman, 1931].

In Table II for the sake of comparison the furfuraldehyde from the Cross and Bevan cellulose fraction has been calculated in all cases as xylan. That it is xylan in every case is an unjustifiable assumption, but it has been shown to be so in the case of the flax, the oat straw and the esparto grass.

Discussion of results.

I. "*Nitrogen factor.*"

In considering the results, the two sets of figures of importance in comparison are those for loss of dry matter, and the respective "nitrogen factors," at the end of 48 days, by which time the decompositions have become very slow. Series A, B and C fall together for comparison, since B consists of the flax shives removed from A in the operation of scutching after the retting process. Retting is, of course, a biological process by which the fibre bundles are loosened from the wood. In the first stage the soluble materials are fermented away by aerobic organisms, producing thereby anaerobic conditions in the tanks and paving the way for the main anaerobic fermentation of

Table III. *"Nitrogen factor" of various materials at several stages of decomposition.*

Series No.	Plant material	Time in days	Total N %	NH_3-N %	Organic N %	Loss of dry matter %	Organic N per 100 g. original	"Nitrogen factor"	Nitrogen equivalent
A	Flax straw (normal)	—	0·51	—	0·51	—	—	—	—
		8	1·46	0·15	1·31	19·54	1·05	0·54	2·76
		16	1·50	0·07	1·43	24·22	1·08	0·57	2·35
		24	1·68	0·17	1·51	31·10	1·04	0·53	1·70
		48	1·91	0·22	1·69	42·62	0·97	0·46	1·08
B	Flax shives (retted)	—	0·21	—	0·21	—	—	—	—
		8	0·54	0·19	0·35	5·14	0·33	0·12	2·33
		16	0·53	0·19	0·34	12·90	0·30	0·09	0·70
		24	0·70	0·31	0·39	16·76	0·32	0·11	0·66
		48	0·77	0·36	0·41	19·63	0·33	0·12	0·61
C	Flax fibre	—	0·55	—	0·55	—	—	—	—
		8	0·88	0·19	0·69	1·96	0·68	0·13	6·63
		16	1·28	0·29	0·99	9·16	0·89	0·34	3·71
		24	1·22	0·21	1·01	18·98	0·82	0·27	1·42
		48	1·48	0·27	1·21	28·29	0·87	0·32	1·13
D	Willow peelings	—	1·58	—	1·58	—	—	—	—
		8	2·67	0·32	2·37	2·77	2·30	0·72	26·0
		16	3·01	0·52	2·49	6·54	2·32	0·74	11·3
		24	3·05	0·32	2·73	14·20	2·34	0·76	5·3
		48	3·34	0·26	3·08	15·39	2·61	1·03	6·7
D2*	Willow peelings (without added nitrogen)	8	1·82	Nil	1·82	6·20	1·70	0·12	(1·93)
		16	2·01	Nil	2·01	12·22	1·76	0·18	(1·47)
		24	2·22	0·06	2·16	15·33	1·83	0·25	(1·63)
		48	2·21	0·05	2·16	16·93	1·79	0·21	(1·24)
E	Oat straw	—	0·30	—	0·30	—	—	—	—
		8	1·91	0·71	1·20	12·58	1·05	0·75	5·96
		16	1·56	0·40	1·16	24·90	0·97	0·67	2·69
		24	1·92	0·25	1·67	37·03	1·05	0·75	2·03
		48	2·54	0·36	2·18	50·44	1·10	0·80	1·38
F	Oat straw extracted with hot water	—	0·27	—	0·27	—	—	—	—
		8	1·65	0·86	0·79	2·05	0·77	0·50	24·4
		16	1·61	0·81	0·80	10·02	0·72	0·45	4·41
		24	1·53	0·77	0·76	13·82	0·66	0·39	2·82
		48	1·71	0·74	0·97	17·79	0·80	0·51	2·86
G	Oat straw extracted with 4 % NaOH	—	0·12	—	0·12	—	—	—	—
		8	1·70	1·51	0·19	8·86	0·17	0·05	0·45
		16	1·49	1·27	0·22	14·53	0·19	0·07	0·48
		24	1·18	0·82	0·36	16·84	0·30	0·18	1·07
		48	0·88	0·53	1·35	21·32	0·28	0·16	0·74
H	Oat straw extracted with 2 % HCl	—	0·34	—	0·34	—	—	—	—
		8	1·64	1·24	0·40	4·24	0·38	0·04	0·94
		16	1·67	1·29	0·38	6·76	0·35	0·01	0·14
		24	1·43	0·76	0·67	8·32	0·61	0·27	3·24
		48	1·11	0·44	0·67	9·14	0·61	0·27	2·95
I	Oat straw extracted with 4 % alc. NaOH	—	0·11	—	0·11	—	—	—	—
		8	1·26	0·88	0·38	11·24	0·34	0·23	2·05
		16	1·28	0·94	0·34	16·72	0·28	0·17	1·02
		24	1·47	0·97	0·50	19·26	0·40	0·29	1·31
		48	0·98	0·39	0·59	21·11	0·47	0·36	1·70
J	Barley straw extracted with 4 % NaOH	—	Nil	—	Nil	—	—	—	—
		8	1·22	0·95	0·27	15·16	0·23	0·23	1·52
		16	1·08	0·60	0·48	17·67	0·39	0·39	2·20
		24	1·20	0·56	0·64	18·81	0·52	0·52	2·76
		48	0·76	0·21	0·55	19·66	0·44	0·44	2·24
K	Esparto grass waste	—	0·95	—	0·95	—	—	—	—
		28	2·03	0·14	1·89	21·93	1·48	0·53	2·41
		56	2·05	0·07	1·98	25·65	1·47	0·52	2·03
L	Esparto grass waste extracted with 4 % alc. NaOH	—	0·23	—	0·23	—	—	—	—
		28	1·13	0·41	0·72	23·20	0·55	0·32	1·38

* Nitrogen absorbed as ammonia from incubator.

pectin which results in the separation of the bundles. In the flax shives, therefore, there must be an absence of soluble and easily fermentable material, and a close agreement in the contents of the main structural constituents, since these are unaffected in the retting process. The figures obtained on the rotted materials show a very marked difference, considerably larger than anticipated in view of the resemblance in composition. The straw suffered a loss of over 40 % and the shives less than 20 %, the nitrogen immobilised by the latter being only one-fourth of that locked up by the former. This would suggest that the absence of readily soluble and fermentable material tends to lower the "nitrogen factor." The flax fibre, which is completely deficient in soluble materials and consists mainly of cellulose, suffers a 30 % loss, at the same time immobilising 0·32 % of nitrogen. The fibre being non-lignified is in theory wholly available, but nevertheless decomposition is not extensive nor nitrogen immobilisation large. Possibly this is due to the fact that the range of active organisms is rather restricted since the medium is almost completely cellulosic.

The same general trend will be seen in the oat straw series, E–I. The loss of organic matter in the case of water-extracted straw is unaccountably low. The hemicellulose content of this material is high, and from the analytical figures a decomposition considerably more extensive would have been expected. In spite of this, however, the "nitrogen factor" is fairly high, being 0·5 as opposed to 0·8 in the case of normal straw. In Series G the straw was extracted extensively with 4 % NaOH, and this is seen to have the effect of removing the major part of the hemicelluloses, as evidenced by the low pentose and uronic acid figures, while in addition the lignin content is considerably lowered. The composition of the resulting material approaches that of the flax fibre, the differences being that it still contains pentose material intimately associated with the cellulose and is partially lignified. The absence of soluble materials and the presence of lignin together limit the extent of decomposition to a little over 20 % and the "nitrogen factor" to the low figure of 0·16. This may also be seen in Series H, in which all soluble and hydrolysable material was removed by boiling for a considerable period with 2 % HCl, a process which does not remove lignin to any extent. The final composition of this extracted material resembles that of certain woods. The total loss of organic matter did not reach 10 % but the "nitrogen factor" was a little higher than expected, being 0·27. In Series I, the oat straw was extracted with 4 % NaOH in 55 % alcohol, a treatment designed to remove much of the lignin without extracting the hemicelluloses. The lignin content was lowered from 18·5 to 8·2 %, but a portion of the hemicelluloses was also removed. In later work, the NaOH concentration was lowered to 2 % and the alcohol concentration increased to 60 %. The decomposition after this treatment was rather more than 20 % and the "nitrogen factor" 0·36. It was noticed that this treatment resulted in an unusual physical condition of the straw, and, however prolonged the washing with alcohol and cold water, the straw remained very gelatinous when wet.

Series J was a young barley straw cut 10 weeks after the appearance of the first tiller and exhaustively extracted with 4 % NaOH, a procedure which resulted in the production of a material giving analytical results very similar to those of the flax fibre. The figures on decomposition also resembled those of flax fibre, there being a 20 % decomposition and a "nitrogen factor" of 0·4.

To examine again the effect of extraction with alcoholic NaOH and the result of lowering the lignin content by this means, esparto grass waste was treated in this way. It will be seen in K and L that the loss of organic matter in the treated and untreated is about equal, but that the "nitrogen factor" is lowered in the former case from 0·5 to 0·3. That decomposition is not more extensive is no doubt due to the physical condition of the extracted material.

All the above mentioned materials contained an insufficient supply of nitrogen as evinced by their positive "nitrogen factors"; in the absence of added nitrogen their decomposition would have been slow or in some cases negligible in extent. The willow peelings, however, must be considered separately. In Table I a "nitrogen factor" of 1·33—the highest ever observed—was recorded for a sample of this material. This particular sample contained originally 1·9 % N, an amount so large that it would be expected to be more than sufficient for complete decomposition. In fact, it might be expected that losses of nitrogen would occur during the rotting process. Nevertheless, in the presence of added inorganic nitrogen an additional amount of 1·33 % per 100 g. was retained. A second sample, distinctly more woody in nature, was obtained, the analytical figures for which are given under D in Table II. It will be seen that the lignin estimation by the 72 % H_2SO_4 method gave 36·5 %. On decomposition in the presence of added ammonia, just over 15 % of organic matter was lost and 1·0 g. nitrogen immobilised per 100 g. In the absence of added nitrogen rather more organic matter was lost. There was, however, a slight pick-up of nitrogen from the ammonia lost by the other bottles in the incubator. This, however, does not obscure the salient point, which is that this material, though already containing ample nitrogen for decomposition will cause the immobilisation of a considerable additional amount without, however, undergoing a more extensive decomposition. In fact, the presence of ammonia seems even to have retarded decomposition in the early stages. It is clear that the protein or nitrogenous material of the willow peelings, although available, is less so than the ammonia, which is utilised preferentially.

Similar observations have been made in the case of certain other substances given in Table IV.

The retarding effect of an unnecessary addition of nitrogen in the form of ammonium carbonate is seen in the case of the maize straw decompositions, while actual losses, shown by a negative "nitrogen factor" occur in the case of the bean husks which have a very high initial nitrogen content.

The change in the "nitrogen factor" as the decomposition proceeds is of

Table IV. *"Nitrogen factors" of certain materials already containing sufficient nitrogen for decomposition.*

Plant material		Original nitrogen content %	Organic nitrogen content of rotted material %	Loss of organic matter %	"Nitrogen factor"
Rice straw (abnormal manurial treatment)	No N	0·31	0·59	25·36	0·13*
	With N	0·31	1·37	25·37	0·63
Maize straw (Brazil)	No N	1·02	1·61	36·7	—
	0·5 % N	1·02	1·79	31·53	0·20
	1·0 % N	1·02	1·86	28·31	0·31
	2·0 % N	1·02	2·21	26·20	0·28
Bean husks:					
Sample I	With N	3·00	6·48	44·08	0·62
Sample II: (*a*)	No N	2·99	4·80	48·31	−0·51
(*b*)	No N	2·99	4·64	47·16	−0·54
(*a*)	With N	2·99	5·40	42·54	0·11
(*b*)	With N	2·99	5·38	40·93	0·18

* Absorbed from ammonia in incubator.

some interest, since it emphasises the fact that the nitrogenous figures represent an equilibrium between immobilisation on the one hand and ammonification on the other. The equilibrium point may change during the course of a decomposition, for as the various plant constituents are removed and by-products formed, there is a sequence of active forms. Under the conditions of the experiments given above, relative stability would have been reached before the end of 48 days. A higher "nitrogen factor" may be recorded earlier as shown in Series A (flax straw) or in other cases there may be a fall followed by a rise.

II. *"Nitrogen equivalent."*

The "nitrogen factor" is an expression which contains no direct reference to the efficiency of the organism or organisms in decomposing the plant material in question, or inversely, to the availability of the material. Two plant materials may be found to have the same "nitrogen factor," say 0·5, but one may have lost in decomposition 50 % and the other only 20 %. Obviously the organisms in the former case were more efficient per unit of nitrogen, the plant material being readily available. If, however, the nitrogen requirements are re-calculated on a basis of equal amounts of organic material fermented away, a figure is obtained which measures the efficiency of the microbial tissue in decomposition. The term "nitrogen equivalent" is suggested for this factor, some of the theoretical aspects of the use of which have been described in Part IV of this series. In the example given above, the former material would have a "nitrogen equivalent" of 1·0 and the latter of 2·5, indicating the difference in availability of the two materials. The "nitrogen equivalent" may be defined as the nitrogen immobilised in the course of the removal of 100 g. of organic matter from any material. When determined at any given time of decomposition, this factor also is a summation of the

microbial activities up to that point. If the decomposition is effected by a mixed general flora, ammonifying organisms by liberating ammonia from dead microbial tissue undoubtedly reduce the total immobilised nitrogen and render both the "nitrogen equivalent" and the "nitrogen factor" apparent rather than absolute. If rapid ammonification takes place it is possible that a portion of the nitrogen may be active, that is, present as living protein in active microbial tissue more than once during the decomposition. This, however, does not wholly invalidate the use of the "nitrogen equivalent" for mixed flora decompositions, though it must be admitted that its chief value is for pure culture decompositions. As a standard for comparison the figures for Series E, oat straw, may be taken, the initial "nitrogen equivalent" being about 6, falling after 48 days to 1·4. The following figures for rice straw were calculated from those given by Rege [1927] and show the same trend in more detail.

Table V. *"Nitrogen equivalent" of rice straw in decomposition (Rege).*

Time in days	4	8	12	16	20	24	28	36	40
Loss of dry matter (%)	4·4	8·8	24·6	30·2	36·1	39·2	41·4	43·2	45·0
"Nitrogen factor"	0·12	0·53	0·88	0·90	0·92	1·02	0·93	0·91	0·90
"Nitrogen equivalent"	2·71	6·01	3·58	2·98	2·55	2·60	2·24	2·10	2·00

The standard "nitrogen equivalent" likely to be reached by mixed flora decomposition of a common straw is about 1·5, it being assumed that it has a "nitrogen factor" of 0·75 and suffers a loss of organic matter of 50 %.

The effect of extraction of the several plant materials on the "nitrogen equivalent" does not appear to be so regular as in the case of the "nitrogen factor." The removal of easily soluble materials in the case of the straw extracted with hot water caused a high initial "nitrogen equivalent" falling at the close to about 3, a figure distinctly higher than that for the normal straw. In the case of the flax shives the initial "nitrogen equivalent" is only a little over 2, falling ultimately to 0·6. There is similarly a considerable difference in trend between the highly cellulosic materials in Series C, G and J, indicating differences in availability which cannot be assessed by any method of chemical analysis.

Conclusions.

To determine the relationship, if any, between the chemical composition of a material and the quantity of inorganic nitrogen temporarily immobilised during decomposition was the purpose of these experiments, and in this only partial success has been achieved. It is possible to indicate approximately what the "nitrogen factor" is likely to be, knowing the general composition of the material, but any absolute prediction is not possible for at least two reasons. In the first place the mechanical and physical differences in structure cannot be determined or taken into account, but must affect considerably the biological availability of the tissue. In the second place, the nitrogen picture is obscured by the fact that it is only possible to determine the changes

in the quantity of added inorganic nitrogen immobilised. It is impossible to learn whether the natural proteins of the plant tissue are also utilised. The evidence in the case of Series D, willow peelings, would suggest that in the presence of a sufficiency of inorganic nitrogen, they are not utilised, but there is no means of being certain on this point. It is possible that plant proteins are as readily ammonified as microbial tissue and that in the presence of ammonifiers, the normal condition in the case of a mixed flora, the ammonia liberated would be utilised by other organisms. If this is the case, the actual total "active" nitrogen would be considerably in excess of the "nitrogen factor" and the latter would merely be the difference between the amount of nitrogen liberated from the plant proteins and the total nitrogen requirement of that plant material in decomposition. If that is so, then the amount and availability of the nitrogenous substances of the plant is of great significance in determining the "nitrogen factor." As "availability" is impossible to ascertain, any prediction on these grounds is of doubtful value.

It seems that a high "nitrogen factor" is likely to be given by a substance containing about 50 % Cross and Bevan cellulose, not more than 20 % lignin, a fairly high content of hemicelluloses as measured by free pentose content, and a low nitrogen content, provided that it also contains water-soluble material. The removal of the water-soluble material seems markedly to affect the "nitrogen factor," and plant wastes that have been submitted to extraction with water or removal of these substances by biological means, although otherwise suitable, are likely to have a "nitrogen factor" considerably lower. A highly cellulosic material low in nitrogen will have a "nitrogen factor" of 0·3 to 0·4, while high lignification depresses the factor, since decomposition is impeded.

Summary.

1. No direct relationship was found between the composition of plant materials and the amount of additional available nitrogen immobilised during decomposition ("nitrogen factor").

2. The nitrogen factor represents only the equilibrium between immobilisation on the one hand and ammonification on the other, and does not necessarily represent the whole of the nitrogen active in decomposition, since the plant proteins may also be attacked and utilised in part, or microbial nitrogen may be liberated and re-utilised.

3. Plant materials already containing sufficient or more than sufficient nitrogen for decomposition may nevertheless immobilise an additional amount, owing to preferential utilisation of the inorganic form.

4. If the nitrogen requirements of materials in decomposition are related on the basis of equal amounts of organic matter removed, a figure is obtained which expresses the efficiency of the organism or mixed flora effecting the decomposition. This "nitrogen equivalent," like the nitrogen factor is a summation of the microbial activities and is apparent rather than absolute.

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CXCII. THE BIOLOGICAL DECOMPOSITION OF PLANT MATERIALS.

VI. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE RATE OF IMMOBILISATION OF NITROGEN BY STRAW.

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The total nitrogen requirements of any plant material in decomposition may be determined quite simply, and can conveniently be expressed by some such term as the "nitrogen factor." This term was first employed by Hutchinson and Richards [1921], and may be defined as the inorganic nitrogen temporarily immobilised by 100 g. of material in decomposition. Certain considerations which affect the nitrogen factor have been discussed in the preceding paper of this series [Richards and Norman, 1931]. The rate of immobilisation has, however, not been determined, nor has the influence of hydrogen ion concentration upon the rate been investigated. Any differences either in rate or extent of decomposition which may be observed will, of course, be due to a modification of the active flora. It might be expected, therefore, that both the rate of fermentation and the amounts of the various constituents removed might vary considerably with changes in the p_H of the medium.

It was necessary in the first place to devise some means by which the hydrogen ion concentration of the decomposing material could be kept approximately constant, and by which a sufficiency of available nitrogen would be present at all stages of the decomposition, without having a large excess in the initial stage since this might interfere with the normal course of decomposition. Both these requirements pointed to some arrangement for the continuous supply of nitrogen.

Richards showed that a solution of ammonium carbonate (10 parts of nitrogen per 100,000), could be passed through a percolating filter of wheat straw until, on the twentieth day, only 1 % of the ammonia nitrogen would be found in the effluent and all but a small percentage (3·4) of the balance retained in the filter as microbial protein. From the twentieth day the mature filter would continue to remove practically all the nitrogen fed to it until saturation point was reached, *i.e.* until the "nitrogen factor," 0·7 per 100 parts of dry straw, had been attained. This laboratory experiment and the subse-

quent successful trials on the large scale made by Richards and Weekes [1921] with sewage were carried out at a p_H of 7·0–8·0.

Accordingly straw was arranged in the form of a filter and supplied with a very dilute solution of an ammonium salt adjusted to the required p_H. This appeared to give very satisfactory results, and is a method suitable for other studies of a similar nature.

Experimental.

Known amounts of dry chaffed straw were packed into a series of lamp glasses as supplied for paraffin lamps, the wider end being covered by muslin held in place by rubber bands. The most convenient size of lamp glass held about 50 g. straw, but others rather larger holding 70 g. were also employed and were quite satisfactory.

A dilute solution of an ammonium salt together with certain mineral salts, adjusted to the required hydrogen ion concentration, was allowed to drop through at the rate of 1 litre per day. At the concentration of nitrogen employed (7 parts per 100,000) this was sufficiently in excess for all stages of the decomposition. Even at the most active point less than 50 % of the nitrogen was retained while in general 10 to 15 % only was immobilised. The mineral salts were added in case the leaching effect of a continuous flow removed any essential ions. The potash, for example, is easily washed out of straw[1]. The rate of flow maintained was sufficient to keep the p_H close to the desired point, and quite insufficient to cause the development of anaerobic conditions. The distribution was found to be very satisfactory after the first two or three days. By means of the Nessler reaction the amount of ammonia in the effluent was checked daily. This was merely a precautionary measure to ensure the presence of sufficient nitrogen. The results obtained were not sufficiently accurate to be regarded as a measure of immobilisation, since they do not show the amount biologically immobilised by conversion to microbial protein, but merely the amount retained or lost by the filter.

At appropriate intervals, as indicated roughly by the Nessler figures, a whole filter was taken down and the following determinations made:

1. Loss of dry matter.
2. Total nitrogen content—Kjeldahl.
3. Ammoniacal nitrogen content—distillation with MgO.

After drying and grinding, the following determinations were made:

4. Total furfuraldehyde yield.
5. Cellulose—Cross and Bevan.
6. Furfuraldehyde from Cross and Bevan cellulose.
7. Carbon dioxide yield, uronic acids.

[1] Over 90 % of the total K_2O in wheat straw is soluble in water, and in barley straw 97 %. (Richards and Rege, unpublished.)

From the first three the nitrogen factor and nitrogen equivalent of the straw at any point could be calculated and compared with the losses sustained by the various constituents.

Four series of experiments were carried out, the variations of ingoing nitrogenous solution being indicated below. It was not found possible to hold the p_H of the solutions more closely to the desired figure than the limits given below, since it was undesirable to introduce buffering salts.

Series I. $p_H = 8{\cdot}5$–$8{\cdot}8$, N as ammonium carbonate.
,, II. $p_H = 7{\cdot}4$–$7{\cdot}8$, N as ammonium chloride + NaOH.
,, III. $p_H = 5{\cdot}0$–$5{\cdot}5$, N as ammonium chloride + 2 cc. acetic acid per litre.
,, IV. $p_H = 7{\cdot}4$–$7{\cdot}8$, N as ammonium chloride + sucrose to give 26 parts carbon per 100,000 + NaOH.

In each case the necessary amount of ammonium salt to give 7 parts per 100,000 on dilution was dissolved in 1 litre of distilled water together with the following mineral salts:

K_2HPO_4	...	...	2·0 g.
$MgSO_4$	...	...	1·0 g.
NaCl ...	...	...	0·2 g.
$FeCl_2$ ...	...	...	0·05 g.

The p_H was adjusted so that when 10 cc. of the above solution were diluted to 1 litre the required p_H was given. This was a matter of some difficulty, since the distilled water was usually slightly acid and showed some variation, thereby accounting for the variations recorded above. The p_H of the effluent was determined from time to time. These figures will not be given in detail for their absolute value is doubtful. The following are the extreme limits of variation recorded. Usually the figure was nearer that of the ingoing solution. The alkaline filters tended to become more nearly neutral, the neutral filters slightly acid, and the acid filters less acid, though never reaching neutrality.

Series I. Effluent $p_H = 7{\cdot}8$.
,, II. ,, $p_H = 6{\cdot}8$.
,, III. ,, $p_H = 6{\cdot}6$.
,, IV. ,, $p_H = 6{\cdot}4$.

When thoroughly wet each filter was inoculated with 100 cc. inoculum from soil containing decomposing organic matter.

The results of analyses are given in the following Tables. Table I shows the nitrogenous changes at various periods for the four series; Table II contains the analytical figures calculated on the basis of 100 g. of original straw.

Table I. *Nitrogen content of straw filters.*

Day	Total N	Ammoniacal N	Organic N	Loss of dry matter	Organic N on 100 g. original straw	"Nitrogen factor"	Nitrogen equivalent
			Series I.				
—	0·302	—	0·30	—	—	—	—
6	0·47	0·03	0·44	3·19	0·43	0·13	4·07
12	0·70	0·07	0·63	3·88	0·60	0·30	7·73
16	0·80	0·10	0·70	5·10	0·66	0·36	7·06
19	0·85	0·09	0·76	11·00	0·68	0·38	3·45
23	0·87	0·12	0·75	19·77	0·60	0·30	1·52
28	0·75	0·12	0·63	21·04	0·50	0·20	0·95
34	0·82	0·13	0·69	22·69	0·53	0·23	1·01
38	0·83	0·11	0·72	24·56	0·54	0·24	0·98
42	0·98	0·11	0·87	27·91	0·63	0·30	1·07
72	1·90	0·16	1·74	48·08	0·90	0·60	1·25
			Series II.				
—	0·302	—	0·30	—	—	—	—
10	0·59	0·04	0·55	10·38	0·49	0·19	1·82
17	0·77	0·03	0·74	16·57	0·62	0·32	1·93
23	0·90	0·03	0·87	19·15	0·70	0·40	2·06
29	1·07	0·02	1·05	27·37	0·76	0·46	1·68
35	1·15	0·10	1·05	28·10	0·76	0·46	1·64
51	1·28	0·11	1·17	31·81	0·81	0·51	1·60
72	1·38	0·08	1·30	40·67	0·77	0·47	1·16
			Series III.				
—	0·302	—	0·30	—	—	—	—
10	0·57	0·03	0·54	9·74	0·49	0·19	1·99
19	0·69	0·05	0·64	15·22	0·54	0·24	1·57
23	0·74	0·04	0·70	16·98	0·58	0·28	1·65
29	0·80	0·05	0·75	20·91	0·59	0·29	1·38
37	0·90	0·04	0·86	23·48	0·66	0·36	1·53
51	0·85	0·08	0·77	30·84	0·53	0·23	0·74
72	1·15	0·07	1·08	37·48	0·68	0·38	1·01
			Series IV.				
—	0·302	—	0·30	—	—	—	—
10	0·540	0·04	0·50	4·37	0·48	0·18	4·12
17	0·80	0·05	0·75	7·15	0·70	0·40	5·60
23	0·96	0·07	0·89	14·69	0·76	0·46	3·17

Discussion of results.

(*a*) *Nitrogen immobilisation.* Differences in the rate and extent of immobilisation of nitrogen in each series may most readily be seen by comparing the "nitrogen factor" at each stage, since by this is expressed the inorganic nitrogen transformed to organic on the basis of 100 g. of original straw. Comparing first Series II and III, the neutral and acid filters respectively, it is seen that over the first 10 or 12 days very similar amounts are retained, after which period rather more is immobilised under neutral conditions. The neutral filter attains a nitrogen factor of 0·51 after 51 days falling off slightly to 0·47 at the end of 72 days. The acid filter shows a peak of 0·36 in 35 days and after a slight loss finally reaches 0·38 at the end of 72 days. Neutral conditions, therefore, result in the retention of rather more nitrogen than acid. Under slightly alkaline conditions immobilisation is more rapid during the first fortnight than in either acid or neutral conditions. A peak of 0·38

Table II. *Analyses of straw filters.*

(Expressed on 100 g. original dry straw.)

Day	Loss of dry matter	Total furfurald. yield	C. and B. cellulose	Furfurald. yield from C. and B. cellulose	CO_2 yield	Total uronic acid anhydride	Furfurald. from uronic acid anhydride	Furfurald. from pentose in hemicellulose	Xylan associated with C. and B. cellulose	"Pure" cellulose by difference
					Series I.					
—	—	14·95	48·92	6·61	1·36	5·44	0·91	7·43	10·25	38·67
16	5·10	14·15	48·23	6·45	1·21	4·84	0·80	6·90	10·0	38·2
19	11·00	12·69	43·61	6·01	0·86	3·44	0·58	6·10	9·3	34·3
23	19·77	11·90	40·01	4·89	0·84	3·36	0·56	6·45	7·6	32·4
34	22·69	11·15	37·62	4·54	0·85	3·40	0·57	6·04	7·0	30·6
42	27·91	10·19	36·61	4·49	0·85	3·40	0·57	5·13	6·9	29·7
72	48·08	6·04	23·15	2·72	0·60	2·40	0·40	2·92	4·2	18·9
					Series II.					
—	—	14·95	48·92	6·61	1·36	5·44	0·91	7·43	10·25	38·67
10	10·38	13·91	47·87	6·84	0·95	3·80	0·63	6·44	10·6	37·3
17	16·57	11·91	43·88	6·77	0·98	3·92	0·65	4·49	10·5	33·4
23	19·45	11·08	40·20	5·87	0·99	3·96	0·66	4·55	9·1	31·1
29	27·37	10·10	36·70	5·43	0·93	3·72	0·62	4·05	8·4	28·3
35	28·10	9·99	36·15	4·85	0·86	3·44	0·58	4·56	7·5	28·6
51	31·81	9·09	33·83	4·54	0·77	3·08	0·51	4·04	7·0	26·8
72	40·67	7·18	24·84	3·34	0·76	3·04	0·50	3·34	5·2	19·6
					Series III.					
—	—	14·95	48·92	6·61	1·36	5·44	0·91	7·43	10·25	38·67
10	9·74	14·13	48·91	6·80	0·99	3·96	0·66	6·67	10·5	38·4
19	15·22	13·14	43·45	6·55	0·96	3·84	0·64	5·95	10·2	33·3
23	16·98	12·62	42·40	6·33	0·93	3·72	0·62	5·67	9·8	32·6
29	20·91	11·45	38·81	5·37	0·89	3·56	0·59	5·49	8·3	30·5
37	23·48	11·27	37·61	5·02	0·96	3·84	0·64	5·61	7·8	29·8
51	30·84	10·07	33·74	4·54	0·87	3·48	0·58	4·95	7·0	26·7
72	37·48	7·86	27·54	3·79	0·78	3·12	0·52	3·55	5·9	21·6
					Series IV.					
—	—	14·95	48·92	6·61	1·36	5·44	0·91	7·43	10·25	38·67
10	4·37	14·05	49·64	7·09	1·17	4·68	0·78	6·08	10·99	38·7
17	7·15	13·49	47·77	6·32	1·08	4·32	0·72	6·45	9·80	38·0
23	14·69	12·26	44·26	5·72	0·95	3·80	0·63	5·91	8·87	35·4

is reached after 19 days and is followed by small losses. Finally over 72 days as much as 0·6 g. nitrogen is immobilised per 100 g. of straw. Alkaline conditions, therefore, seem ultimately even more favourable than neutral or acid, though in the intermediate period, from 4 to 5 weeks, less may be held than in the latter.

(*b*) *Loss of dry matter.* Under the conditions of the experiments the total loss of dry matter observed is not solely due to biological assimilation. In part it is due to the leaching out of soluble materials of the straw, and in part due to the removal of soluble intermediate products of microbial activity. A filter run solely on water for 51 days showed a total loss of dry matter of 21·26 %. This included biological action for no attempt was made to keep the filter sterile. There was in fact, a small "nitrogen factor" of 0·09 due to the picking up of ammonia from the air.

In the acid and neutral series losses of dry matter are very similar over the first fortnight, after which the neutral filter goes slightly ahead, and during the whole period loses rather over 40 % as opposed to 37 % in the case of the acid filter. Particularly noticeable is the regularity in the rate of decomposition.

In the alkaline filter in the early stages the rate of decomposition is slow, even though considerable immobilisation of nitrogen is taking place, and after 16 days only 5 % of dry matter has been lost. During the following weeks decomposition is very rapid, after which the rate becomes normal again. Over 72 days, 48 % is lost. The most notable feature is the well-established initial lag in decomposition, in spite of which the total final loss is greater than in either the neutral or acid series. Series IV was a short period run with a neutral sugar-nitrogen solution, in order to see how the addition of readily available carbohydrate affects the rate of decomposition of straw and the amount of nitrogen immobilised in the early stages. Even under these conditions with an excess of very readily available carbohydrate material, the loss of dry matter from the straw is as great as in the alkaline run over the same period, though the "nitrogen factor" is a little higher. Acid-production from the sugar is evident from the p_H figures. The addition of the sugar did not result in the immobilisation of as much nitrogen as might have been expected, and the experiment was discontinued.

(*c*) *Nitrogen equivalent.* The differences between the several series are well brought out by the nitrogen equivalent figures. The lower this figure, the less nitrogen has been immobilised in removing 100 g. of carbohydrate material from the straw. Both the neutral and the acid filters have similar nitrogen equivalents, that of the acid being just a little lower. The initial lag in decomposition in the alkaline filter is due to the unsuitability for cellulose decomposition of the flora developing in the opening stages, shown by the fact that the nitrogen equivalent is much higher then, being well over 7 as opposed to a maximum figure of 2 in either of the other series.

(*d*) *Loss of various constituents.* A comparison of the rate of loss of certain structural constituents shows no unusual features. The final loss of cellulose is rather greater in the alkaline filter than in either of the others as would be expected from the losses of organic matter observed. There is, however, an initial lag in the alkaline filter and even at the end of 6 weeks less has been removed than in the others. The loss of hemicellulose as measured by the disappearance of pentose presents precisely similar features, but is very different from that found to occur in straw compost heaps. Under such circumstances a very rapid fermentation of the hemicelluloses occurs in the early stages, before cellulose decomposition is appreciable. Later while the cellulose suffers a heavy loss, the hemicelluloses appear to remain at much the same level. In the filter it is true that decomposition of pentose precedes that of cellulose, but in no case does it exhibit an early peak, and losses continue at a fairly steady rate side by side with cellulose degradation

throughout the period of the experiments. The changes in the neutral filter approach most closely to those observed in compost heaps, in that the early loss of hemicelluloses is considerable but this loss is neither so large nor so rapid as would be found in the latter. The loss of xylan associated with the cellulose seems to follow closely the loss of the cellulose itself, as would be expected from its distribution.

Conclusions.

From the results presented it seems that changes in hydrogen ion concentration exercise a considerable effect on the rate and amount of nitrogen immobilised by straw undergoing decomposition. It is especially to be noted, however, that the total amount of organic matter lost may not be very different, nor the relative losses of individual constituents much affected by such changes. The wide range of organisms present in the soil and the co-operative nature of their activities make possible a large number of balanced reactions, each capable of bringing about the same ultimate result, though the route and rate of achieving it may differ. The effects and differences observed are undoubtedly due to a modification in nature of the active microflora. In the case of the acid filter, the decomposition of the straw was mainly the work of fungi, the growth of which would be favoured by such conditions; indeed a heavy growth of fungal tissue, mainly *Trichoderma*, was clearly visible in the earlier stages. There is probably a sequence of active forms as the straw undergoes modification in decomposition. The nitrogen factor in this series showed a peak of 0·36 after 5 weeks, followed by a slight fall and rise again to 0·38. The fall observed is direct evidence for the ammonification that proceeds side by side with the immobilisation of nitrogen. Ammonification may be due either to bacteria or fungi, but probably the latter. In the neutral filter the conditions are more suitable for bacterial development and not unfavourable to fungi. It is therefore not surprising that the nitrogen factor is consistently higher than under acid conditions since, in general, bacteria have a higher nitrogen content than the fungi. It is not unlikely that the fungi carry out the major part of the cellulose decomposition and that the bacteria are active in removing degradation products and synthesised fungal tissue.

Under slightly alkaline conditions it appears that the development of the fungi is hindered, and that, since the fungi are the more active in cellulose degradation, there is, in consequence, an initial lag in decomposition. It is possible that it is necessary for the conditions in the filter to become more nearly neutral before the fungi develop and that this is effected by the action of bacteria producing acid from the soluble constituents. The sudden rapid loss of organic matter which occurs at the end of a fortnight is accompanied by a considerable fall in nitrogen factor and this can be explained by fungal ammonification of the bacterial film built up in the early stages. The nitrogen

equivalent figures at this period are very significant, since they reveal the fact that the flora present suddenly becomes much more active in decomposition, implying a change of type of organism. Manure and compost heaps undergoing natural aerobic decompositions generally remain fairly alkaline or neutral, so that the maximum immobilisation of nitrogen probably occurs. The practice of adding calcium carbonate to ensure this has clearly much to recommend it, particularly in the case of composts to which ammonium sulphate has been added. However, it sometimes happens that through waterlogging or too close packing, anaerobic or partially anaerobic conditions occur, in which circumstances organic acids are produced from cellulose and the heap becomes definitely acid. Nitrogen may then be lost by ammonification and washing out of ammonium salts or by evaporation in the air. When a source of nitrogen is added in composting, as in the Adco process for the production of artificial farmyard manure, the aim is to immobilise as much nitrogen as possible in a biologically available organic condition. To achieve this it is desirable, therefore, to keep the reaction of the heap on the alkaline side. This was mentioned by Richards and Hutchinson [1924] in their patent, and the biological reasons for it are now made clear in this paper.

It is proposed to extend this work to conditions which occur in abnormal soils of various types, such as alkaline soils, very acid soils, and soils containing considerable quantities of salts.

Summary.

1. By the use of percolating straw filters supplied with available nitrogen at high dilutions, the rate of immobilisation of nitrogen during decomposition at various hydrogen ion concentrations has been determined.

2. Under slightly alkaline conditions immobilisation is more rapid in the early stages than in either slightly acid or neutral conditions, and ultimately more nitrogen is retained. Neutral conditions seem rather more favourable than acid for immobilisation.

3. In 10 weeks more organic matter is fermented away under alkaline than neutral conditions, and more under neutral than acid conditions. The alkaline filter shows an initial lag in decomposition not given by either of the others. Nitrogen equivalent figures show this to be due to the development of a primary flora relatively inactive as far as decomposition of cellulose is concerned.

4. In each case the loss of hemicelluloses is rather different from that found to occur in compost heaps, it being considerably more gradual in the filters.

5. The effects observed are due to differences in character of the active flora.

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and to the Department of Scientific and Industrial Research for a Senior Research Award, during the tenure of which this work was carried out.

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CXCIII. EXPERIMENTS ON NUTRITION.
XI. COMPARATIVE VITAMIN B_1 VALUES OF FOODSTUFFS. FRUITS AND VEGETABLES.

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The vitamin B or B_1 values of fruits were first investigated by Osborne and Mendel [1919] who found that 5 g. of fresh apples or pears averted a decline in weight of rats, and that 10 cc. of orange juice daily gave good growth. Later [1920], they showed that 10 to 15 cc. daily of orange, lemon or grape fruit juice led to normal growth of rats. The juice of the grape was not good; some growth resulted with 2 to 5 g. of prunes. Apples and pears were then again tested and found to be a poor source of vitamin B. The poor value of grape juice has also been recorded by Randoin [1930]. The banana was shown by Sugiura and Benedict [1918] to be deficient in vitamin B. Miller [1926] found that 15 to 20 g. daily of papaya as source of vitamin B gave fair growth in rats. Experiments with orange juice have also been made by Stammers [1924] and by Willimott [1928], who confirm the earlier results that 10 cc. gave good growth but scarcely equal to the normal. The rind of lemon and grape fruit contained vitamin B as shown by Willimott and Wokes [1926]. House, Nelson and Haber [1929] stated that 3 g. of fresh tomato daily sufficed for maintenance of rats. The only tests on pigeons appear to be those by Chick and Hume [1917] who found that extracts of currants and dates had only a very slight curative effect on pigeons suffering from polyneuritis.

A fairly large number of vegetables have been examined for their comparative values in vitamin B. In all these experiments the dried vegetable was used and growth of rats was the criterion of their value. In the earlier tests the vegetable was incorporated in the food, but later a small quantity of the dried vegetable was given daily, in many experiments by Osborne and Mendel after a decline in growth of the rats had started. The results are summarised in Sherman and Smith's book [1931] and, in order to save space, are not quoted in detail here.

The older standard test, cure or prevention of polyneuritis in pigeons, for the presence of vitamin B_1 so much advocated by Chick [see Chick and Copping, 1930] has not been used since the tests on cabbage and potato by Chick and Hume [1917]. The curative method was then used: cabbage was

found to contain vitamin B, but potato was almost deficient. The preventive method has not hitherto been tried with fruits and vegetables. We have used this method throughout our series of experiments and are now able to report upon a series of about 30 common fruits and vegetables. Some of these tests were made several years ago, but to complete the series and publish as a whole was considered preferable to publishing one or more of the results separately. A clearer picture is thus revealed and reference to the matter is much easier.

Experimental.

The tests on the vitamin B_1 values of fruits and vegetables have been made on pairs of pigeons under the same housing conditions as in our previous experiments with pulses, nuts and cereals [Plimmer, Raymond and Lowndes, 1929, 1931].

It was recognised that the high water content of fruits and vegetables would occasion some difficulty in our method of testing, *i.e.* mixing the foodstuff with white rice or white flour and fishmeal. In a dried form only apple rings and apricots were obtainable; cabbage and other leaves could be dried by exposure to warm air, and roots, like carrots, could be cut up into small cubes. Our trials with these foodstuffs thus prepared and mixed with white rice and fishmeal led to no result, as the pigeons ate the rice and left the fruit or vegetable and suffered from polyneuritis. This experience led to the conclusion that these foods contained very little vitamin B_1, for we had always noticed that pigeons preferred foods containing vitamin B_1 to white rice and generally ate such foods first and left the white rice. Only boiled potato, passed through a squeezer, was readily eaten by the birds. It thus seemed that pigeons preferred fruits and vegetables in the cooked state. Cooked vegetables and fruits were therefore used as the means of procuring consumption. At the same time water could be removed by evaporation. Our procedure was to pass the fruit or vegetable through a mincer, collect all the water and solid matter and heat in a basin on a water-bath until the material became of such a consistency that it could be mixed with flour and fishmeal, and also cod-liver oil in the later tests, to form a dough which could be made into pills and dried. Pills of most of these foods were readily eaten by the pigeons; only onions seemed distasteful and acid fruits, such as rhubarb and gooseberries, were at first not palatable. This procedure was really the most suitable as it was desired to know the value of these foods in a form as close as possible to that in which they are used in human consumption. Practically all vegetables are eaten in the cooked state and fruits are eaten fresh or cooked. No objection can be made to cooking as it has been shown by many workers that vitamin B_1 is not destroyed by a temperature of 100°. All the fruits and vegetables were purchased from a local greengrocer and were the same as those sold for human consumption.

In the tests with cereals and pulses, from 20 to 40 % had to be incorporated in the diet to secure maintenance of pigeons for our test period of 26 weeks.

In the first trials with fruits and vegetables 50 % of the fresh food was mixed with the flour and fishmeal. With most the pigeons showed head retraction in periods varying from 3 to 9 weeks. It was not considered necessary to test the food in a higher proportion except when the period was about 9 weeks; 70 or 80 % was then tried. Potato was tested at 90 and 99 %. The results are shown in the following three tables under fruits; vegetables, leaves; and vegetables, roots and tubers. They are given in alphabetical order and not in the order of testing. The order of testing depended largely upon the season of the year when the material was procurable.

Table I. *Fruits.*

		Diet				Weights, g.		
Date	Time in weeks	Apple	White flour	Fish-meal	Cod-liver oil	Cock No. 98	Hen No. 62	Remarks
16. i. 29						500	385	
to		50	47·5	2·5	0			
13. ii. 29	4					375	270	Hen had head retraction and was cured
to								
27. ii. 29	6					375	—	Cock had head retraction and was cured
						No. 48	No. 30	
26. iii. 30						410	295	
to		80	18·0	1·8	0·2			
30. iv. 30	5					250	205	Cock had head retraction and died
to								
2. vii. 30	14					—	195	Hen died. Ate only from 5 to 10 g. daily during last 9 weeks
		Banana				No. 83	No. 78	
13. iii. 29						405	390	
to		50	47·5	2·5	0			
10. iv. 29	4					320	335	Cock had head retraction and died
to								
15. v. 29	9					—	260	Hen paralysed and died
						No. 93	No. 50	
29. v. 29						395	435	
to		70	28·5	1·5	0			
7. viii. 29	10					240	300	Both showed head retraction. Cock died, hen cured
		Dried Plantain				No. 7	No. 8	
6. i. 26						590	450	
to		95	0	5·0	0			
10. ii. 26	5					500	385	No more food available. Loss of weight indicates insufficient vitamin B
		Currants *red*				No. 330	No. 70	
19. vii. 28						360	350	
to		50	47·5	2·5	0			
15. viii. 28	4					295	275	Cock had head retraction and died
to								
29. viii. 28	6					—	240	Hen had head retraction and was cured
		black				No. 167	No. 60	
17. vii. 29						385	370	
to		70	28·5	1·5	0			
4. ix. 29	7					270	285	Cock had head retraction and died
to								
11. ix. 29	8					—	265	Hen had head retraction and was cured

Table I. *Fruits (contd.)*.

Date	Time in weeks	Diet: Goose-berries	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 330	Weights, g.: Hen No. 35	Remarks
6. vi. 28						395	415	
to		50	47·5	2·5	0			
27. vi. 28	3					355	305	Hen had head retraction and died
to								
4. vii. 28	4					325	—	Cock had head retraction and was cured
		GRAPES				No. 18	No. 39	
23. x. 29						470	480	
to		70	28·5	1·5	0			
27. xi. 29	5					400	390	Cock had head retraction and was cured
to								
18. xii. 29	8					—	345	Hen had head retraction and was cured
		ORANGE JUICE				No. 66	No. 803	
16. i. 29						445	420	
to		50	47·5	2·5	0			Ate very little after 10th week
15. v. 29	17					310	285	Cock had head retraction and was cured
to								
29. v. 29	19					—	205	Hen died
						No. 18	No. 39	
13. iii. 29						490	450	
to		70	28·5	1·5	0			Five eggs laid by hen
17. vii. 29	18					435	375	Lost some weight and recovered it
to								
29. viii. 29	24					460	405	
to								
2. x. 29	29					420	380	
		ORANGE PEEL				No. 69	No. 79	
16. i. 29						400	370	
to		50	47·5	2·5	0			
13. iii. 29	8					255	300	Cock paralysed and died
to								Hen not well and not eating
8. v. 29	16					—	265	Hen died
						No. 98	No. 62	
13. iii. 29						460	360	
to		70	28·5	1·5	0			Both ate very little during whole period.
25. ix. 29	28					325	265	Lost weight in first weeks
		PLUMS				No. 43	No. 128	
29. viii. 28						315	395	
to		50	47·5	2·5	0			
26. ix. 28	4					240	320	Cock had head retraction and died
to								
3. x. 28	5					—	325	Hen had head retraction and was cured
		RHUBARB				No. 83	No. 96	
15. v. 29						400	320	
to		50	47·5	2·5	0			
3. vii. 29	7					250	250	Cock had head retraction and died
to								
17. vii. 29	9					—	220	Hen had head retraction and was cured

Table I. *Fruits (contd.).*

Date	Time in weeks	Diet: Strawberries	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 17	Weights, g.: Hen No. 72	Remarks
27. vi. 28						450	490	
to		50	47·5	2·5	0			
8. viii. 28	6					310	345	Hen had head retraction and was cured. Cock died with large heart
		Tomatoes				No. 62	No. 43	
26. x. 27						425	460	
to		50	47·5	2·5	0			
30. xi. 27	5					345	330	Both showed head retraction and died
						No. 33	No. 36	
30. iv. 30						390	285	
to		80	18	1·6	0·4			Hen laid 15 eggs; deserted
12. xi. 30	28					465	300	
to		70	26·5	2·0	0·5			
17. xii. 30	5					425	295	Cock had head retraction and died
to								
28. i. 31	11					—	235	Hen had head retraction and died

Table II. *Vegetables, leaves,* etc.

Date	Time in weeks	Diet: Fresh Runner Beans	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 23	Weights, g.: Hen No. 72	Remarks
29. viii. 28						385	455	
to		50	47·4	2·6	0			
17. x. 28	7					260	360	Hen had head retraction and died
to								
24. x. 28	8					235	—	Cock had head retraction and was cured
		Cabbage				No. 81	No. 92	
12. xii. 28						380	410	
to		50	47·4	2·6	0			
20. iii. 29	14					295	385	Both birds had head retraction and were cured
						No. 645	No. 50	
25. ix. 29						430	455	
to		70	28·4	1·6	0			
27. xi. 29	9					405	450	Cock had head retraction and was cured
to								
25. xii. 29	13					—	345	Hen had head retraction and died
						No. 92	No. 91	
8. i. 30						445	390	
to		80	18·5	1·5	0			Hen laid 10 eggs; one
4. vi. 30	21					395	360	hatched, squab died, others deserted
to								
16. vii. 30	27					410	360	Both well, but slight loss of weight
		Cauli-flower				No. 92	No. 32	
8. xi. 27						380	370	
to		50	47·4	1·6	0			
22. xi. 27	2					335	345	Hen died
to								
13. xii. 27	5					240	—	Cock died

Table II. *Vegetables, leaves, etc. (contd.).*

Date	Time in weeks	Diet: Celery	White flour	Fish-meal	Cod-liver oil	Weights, g.: Cock No. 17	Hen No. 16	Remarks
19. x. 27						445	430	
to		50	47·5	2·5	0			
16. xi. 27	4					305	285	Hen died
to								
23. xi. 27	5					325	—	Cock had head retraction and died
		Kale				No. 74	No. 75	
12. xii. 28						390	365	
to		50	47·4	2·6	0			
6. ii. 29	8					310	255	Hen was paralysed and died. Cock had head retraction and died
						No. 645	No. 63	
1. v. 29						415	345	
to		70	28·4	1·6	0			
17. vii. 29	11					335	245	Hen had head retraction and was cured
to								
8. viii. 29	14					335	—	Cock had head retraction and was cured
		Lettuce				No. 89	No. 49	
11. vii. 28						410	350	
to		50	47·6	1·4	0			
8. viii. 28	4					325	285	Both had head retraction and were cured
						No. 65	No. 58	
21. v. 30						400	340	
to		80	18·0	1·6	0·4			Two eggs hatched, squabs
16. vii. 30	8					350	360	died
to								
17. ix. 30	17					265	300	Both had head retraction and were cured
		Spinach				No. 24	No. 70	
10. x. 28						380	390	
to		50	47·4	2·6	0			
28. xi. 28	7					330	365	Both birds had head retraction and were cured
						No. 67	No. 50	
7. v. 30						375	320	
to		80	18·0	1·7	0·3			
13. viii. 30	14					230	225	Cock died
to								
20. viii. 30	15					—	215	Hen died
		Sprouts (Brussels)				No. 68	No. 29	
16. xi. 27						455	350	
to		50	47·5	2·5	0			
7. xii. 27	3					415	230	Hen died
to								
14. xii. 27	4					400	—	Cock had head retraction and died
		Water-cress				No. 17	No. 45	
4. vi. 30						495	315	
to		80	18·0	1·7	0·3			
13. viii. 30	10					395	330	Cock lost weight and recovered. Hen laid 4 eggs; deserted
to								
10. xii. 30	27					455	385	
to		70	27·5	2·0	0·5			
18. iii. 31	14					290	280	Cock had head retraction and was cured
to								
20. v. 31	23					—	325	Hen died with large heart

Table III. *Vegetables, roots and tubers.*

Date	Time in weeks	Diet: Artichokes	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 38	Weights, g.: Hen No. 74	Remarks
23. xi. 27 to		50	47·5	2·5	0	450	390	
11. i. 28	7					325	245	Cock died. Hen had head retraction and died
						No. 330	No. 35	
11. i. 28 to		70	28·5	1·5	0	430	440	
28. iii. 28 to	11					410	400	Both looked ill
2. v. 28	16					320	325	Cock had head retraction and was cured. Hen paralysed and was cured
						No. 61	No. 62	
28. xi. 28 to		80	19	1	0	450	425	
13. iii. 29 to	15					325	405	Cock had head retraction and died
5. vi. 29	27					—	325	Hen for last weeks ate from 2 to 16 g. only each day
		Beetroot				No. 26/28	No. 26/30	
14. xi. 28 to		50	47·5	2·5	0	460	520	
19. xii. 28 to	5					385	325	Cock had head retraction and was cured
16. i. 29	9					—	245	Hen had head retraction and died
		Carrots				No. 26	No. 78	
2. i. 29 to		50	47·5	2·5	0	475	455	
30. i. 29	4					320	220	Cock had head retraction and was cured. Hen had head retraction and died
		Leeks				No. 38	No. 44	
31. x. 28 to		50	47·5	3·5	0	345	435	
27. ii. 29 to	17					275	525	Cock died
						No. 72		
20. iii. 29 to	20					400	470	New cock
24. iv. 29 to	25					290	380	Hen had head retraction and died
5. v. 29	27					245	—	Cock was paralysed and died
						No. 337	No. 38	
16. x. 29 to		60	37·5	2·5	0	415	305	
18. xii. 29 to	9					420	305	Cock escaped
15. i. 30	13					—	245	Hen had head retraction and was cured
						No. 84	No. 2	
8. i. 30 to		60	37·5	2·5	0	590	310	
19. ii. 30	7					415	230	Cock had head retraction and died. Hen was paralysed and died

Table III. *Vegetables, roots and tubers (contd.).*

Date	Time in weeks	Diet: Leeks	Diet: White flour	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 19	Weights, g.: Hen No. 20	Remarks
19. ii. 30						450	375	
to		80	18·5	1·5	0			
19. iii. 30	4					490	385	
to		80	18·0	1·6	0·4			Leeks more fibrous
2. vii. 30	19					350	275	Hen had head retraction
to								and was cured
9. vii. 30	20					310	—	Cock had head retraction and was cured
		Onions				No. 38	No. 82	
8. v. 29						375	360	
to		50	47·5	2·5	0			Birds ill in 5th week
17. vii. 29	10					245	255	Hen had head retraction and died. Cock was paralysed and died
		Parsnips				No. 331	No. 28	
21. xii. 27						455	470	
to		50	47·5	2·5	0			
7. iii. 28	11					330	335	Hen was paralysed and
to								died
6. vi. 28	24					245	—	Cock had head retraction and died
						No. 24	No. 70	
2. i. 29						410	435	
to		70	28·4	1·6	0			
6. iii. 29	9					410	450	
to		50	47·5	2·5	0			
27. iii. 29	3					320	345	Hen had head retraction and was cured. Cock was very ill
						No. 81	No. 13	
1. v. 29						380	320	
to		50	47·5	2·5	0			
17. vii. 29	11					245	295	Cock had head retraction
to								and was cured
18. ix. 29	20					—	225	Hen died
						No. 56	No. 14	
31. x. 28						430	430	
to		60	38·0	2·0	0			Started with 2 squabs which died. Hen laid 6 eggs
15. v. 29	28					350	395	Cock had head retraction
to								and was cured
29. v. 29	30					375	365	Both well
						No. 92	No. 91	
1. v. 29						420	385	
to		60	38·0	2·0	0			Two squabs hatched but showed head retraction in 13th week
18. ix. 29	20					315	240	Hen laid 3 eggs. Loss of
to								weight from poor dry parsnips. Recovered on
13. xi. 29	28					410	355	fresh parsnips

Table III. *Vegetables, roots and tubers (contd.).*

Date	Time in weeks	Diet: Potatoes	Diet: White rice	Diet: Fish-meal	Diet: Cod-liver oil	Weights, g.: Cock No. 24/17	Weights, g.: Hen No. 24/16	Remarks
10. ix. 24						385	370	
to		97·3	0	2·7	0			
31. xii. 24	16					385	415	Both well
to		82·5	16·5	1·0	0			
25. iii. 25	12					410	470	
to		79·2	19·8	1·0	0			Four eggs laid by hen
17. vi. 25	12					320	285	
to		98·8	0	1·2	0			
5. viii. 25	7					395	240	Hen died
to								
9. ix. 25	12					240	—	Cock died
						No. 25/8	No. 25/12	
23. ix. 25						385	380	
to		99·0	0	1·0	0			
14. x. 25	3					460	430	
to		82·4	16·5	1·1	0			
3. iii. 26	20					445	440	
to								
12. v. 26	30					395	205	Hen died
to								
14. vii. 26	39					230	—	Cock died
						No. 5	No. 4	
16. vi. 26						405	425	
to		99·0	0	1·0	0			Four eggs during first 5 weeks
3. xi. 26	20					505	445	
to								
23. ii. 27	36					480	430	
			White flour					
						No. 23	No. 50	
5. xii. 28						425	480	
to		70·0	28·5	1·5	0			
30. i. 29	8					340	400	Both had head retraction. Cock died; hen was cured
						No. 37	No. 338	
16. x. 29						420	390	
to		80	17·5	2·5	0			
8. i. 30	12					315	310	Cock had head retraction and died
to								
9. iv. 30	25					—	315	Hen had head retraction and died
						No. 339		
19. ii. 30						365	—	
to								
7. v. 30	11					315	—	Had head retraction and died
							No. 98	
9. iv. 30						—	335	
to								
11. vi. 30	9					—	235	Had head retraction and was cured
						No. 31	No. 32	
11. xii. 29						395	400	
to		90	8·6	1·4	0			
7. v. 30	21					395	405	
to								
4. vi. 30	25					255	370	Cock had head retraction and was cured
to								
25. vi. 30	28					—	310	Hen had head retraction and was cured

Table III. *Vegetables, roots and tubers (contd.).*

Date	Time in weeks	Diet: Swedes	White flour	Fish-meal	Cod-liver oil	Weights, g.: Cock No. 88	Hen No. 407	Remarks
18. i. 28 to 22. ii. 28	5	50	47·5	2·5	0	515 295	405 250	Cock had head retraction and died
7. iii. 28	7					—	235	Hen died
		Turnips				No. 26	No. 8	
24. x. 28 to 21. xi. 28	4	50	47·5	2·5	0	440 315	325 240	Cock had head retraction and was cured
12. xii. 28	7					—	205	Hen died

The birds were cured by giving a dose of 1 g. of wheat germ, or of marmite.

Discussion.

Fruits. In no case could the pigeons be maintained with the fruit composing 50 % of the diet. With 70 % of the diet composed of fruit maintenance of the pigeons was not longer than from 9 to 11 weeks, except in the cases of orange juice and orange peel. With 80 % of tomatoes the pigeons were maintained for our test period of 26 or more weeks.

The maintenance with orange peel was remarkable as the birds lost weight during the first few weeks and kept at this level for the rest of the test period. The birds ate very little during the whole time. Though the test indicates that orange peel is equal to orange juice in vitamin B_1 value, the general appearance of the birds leads to doubt. We would prefer to suggest that the oil in the rind had some medicinal action. In our experience with autopsies of pigeons which have died from too little vitamin B the intestines have contained dark food mixed often with bile. There have usually been signs of decomposing food in the intestines and it is possible that the essential oil of the orange peel has prevented decomposition and the birds have not been poisoned by a toxin. Such poisoning is a possible cause of the symptom of head retraction. Vitamin B_1 acts by preventing the formation of the toxin from stagnant food in the gut. Evans and Lepkovsky [1929] have noted a sparing action of fat on the requirement of vitamin B_1, and Plimmer, Rosedale and Raymond [1927] found that the fat of the diet required balancing by vitamin B. The fat may have acted partly as lubricant preventing stagnation of the food in the gut. Of the various roots which we have tested parsnips have shown the highest value. These vegetables contain a peculiar essential oil. The vitamin B_1 values of fruits are very small.

Vegetables, leaves. There does not appear to be any similarity in the values of the green leaves. Pigeons were maintained on the diet with 80 % of cabbage and 80 % of watercress. Lettuce and spinach at 80 % and 70 % failed to secure maintenance for 26 weeks. Brussels sprouts were not tested

above 50 %, as this amount indicated very little vitamin B_1. Fresh runner beans probably contain the vitamin B_1 in the seed and not in the green pod, which forms the bulk of the edible matter of fresh beans.

In general, vegetable leaves have a better vitamin B_1 value than vegetable roots and fruits.

Vegetables, roots and tubers. Of the roots and tubers tested, artichokes, leeks, parsnips and potatoes contain some vitamin B_1. Beetroot, carrots, swedes and turnips contain very little. Parsnips at the level of 60 % maintained the pigeons, but the value may be due partly to the essential oil.

The experiments with potatoes were rather variable. At first it seemed that the pigeons could be maintained on 80 % with white rice, but on re-testing with white flour 90 % was scarcely sufficient. The potatoes were tested in different years, and it is very likely that vegetables vary slightly according to the season. The water content of vegetables is higher in wet seasons [Plimmer, 1921] and accordingly there may be a variation in vitamin B_1 value. Young and old samples, as observed with parsnips, would also give a variation in vitamin B value. Another variation may arise from the soil in which the vegetables are grown, and whether the soil has been manured or not manured [*cf.* McCarrison, 1926; Rowlands and Wilkinson, 1930], as well as the kind employed.

Regarding the value of vegetables and fruits as a source of vitamin B_1 in the general human dietary, our results do not indicate that they have any important value. In order to supply sufficient vitamin B_1 from 60 to 80 % of the diet must be composed of these foodstuffs in the fresh state, amounts which are impossible for man to consume. On the average the consumption of fruits and vegetables is from 4 to 8 oz.; only potatoes may form a considerable part of the human daily diet (from 1 to 2 lb.). The requirement of man for vitamin B_1 is probably not the same as that of the pigeon, and even if it is the same as that of the rat as estimated by Plimmer, Rosedale and Raymond [1927] the amount of vegetable usually eaten by man is not alone enough for his vitamin B_1 requirement.

Summary.

In comparison with cereals and pulses, fresh fruits and vegetables have a much lower vitamin B_1 value, but if allowance be made for their high water content, the comparison is more favourable. Table IV (p. 1799) shows the water content, percentages of wet and dry material in the diets, maintenance figure (+ or −) and comparative vitamin B_1 values with dried yeast, our standard, cereals and pulses.

It is difficult to compare these figures with those of the other workers with rats. On the whole they correspond fairly closely in relation to dried yeast, being from 1/5th to 1/10th the value of this material [see Roscoe's comparison, 1930].

Table IV.

	Water content %	Percentage amount in diet: Wet	Dry	Maintenance	Comparative vitamin B_1 value: Wet	Dry
Dried yeast	0	.	4	+		100
Cereals	.	.	40	+		10
Pulses	.	.	30	+		13
Fruits:						
Apple	85·3	50	13·0	−		
		80	37·0	−		
Banana	70·8	50	22·5	−		
		70	40·0	−		
Currants, red	85·7	50	12·6	−		
„ black	80·8	70	31·0	−		
Gooseberries	89·3	50	10·0	−		
Grapes	84·7	70	26·0	−		
Orange juice	87·0	50	11·5	−		
		70	23·0	+	6	20
„ peel	78·9	50	18·0	−		
		70	33·0	+	6	13
Plums	85·7	50	13·0	−		
Rhubarb	94·4	50	5·0	−		
Strawberries	90·1	50	9·0	−		
Tomatoes	93·5	50	6·0	−		
		70	14·5	−		
		80	20·7	+	5	20
Vegetables, leaves:						
Beans, runner	93·2	50	12·0	−		
Cabbage	88·0	50	11·0	−		
		70	22·0	−		
		80	32·4	+	5	13
Cauliflower	90·2	50	16·4	−		
Celery	93·7	50	11·0	−		
Kale	83·3	50	14·4	−		
		70	28·0	−		
Lettuce	95·4	50	4·4	−		
		80	15·6	−		
Spinach	92·8	50	6·7	−		
		80	22·5	−		
Sprouts	88·3	50	11·0	−		
Watercress	93·9	70	12·5	−		
		80	20·0	+	5	20
Vegetables, roots etc.*:*						
Artichokes	79·7	50	17·0	−		
		70	32·0	−		
		80	45·6	+	5	9
Beetroot	90·2	50	17·0	−		
Carrots	87·0	50	11·5	−		
Leeks	85·3	50	12·9	−		
		60	18·0	−		
		80	37·0	+	5	10
Onions	94·2	50	5·5	−		
Parsnips	72·6	50	21·3	−		
		60	35·0	+	7	12
Potatoes	80·5	70	31·0	−		
		80	44·0	−		
		90	64·0	+	4	7
Swedes	88·8	50	10·0	−		
Turnips	93·0	50	6·5	−		

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CXCIV. THE SIGNIFICANCE OF THE MICHAELIS CONSTANT FOR SACCHARASE.

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Michaelis and Menten [1913] suggested that the relation between the rate of hydrolysis of sucrose by saccharase and the substrate concentration could be best interpreted if it was assumed that one molecule of enzyme combined with one molecule of substrate to form a complex, which broke down into free enzyme and fructose and glucose. Adopting this schema Briggs and Haldane [1925] showed that the rate of hydrolysis (A) should be related to the concentration of enzyme (E) and the concentration of substrate (S) by the following expression

$$A = \frac{k_2 E.S}{S + (k_2 + k_3)/k_1},$$

where k_1 is the velocity constant of combination of free enzyme and substrate, k_2 of the reversion of the complex to free enzyme and substrate, and k_3 of the breakdown of the complex into free enzyme and products. The fundamental difference between this expression and that suggested by Michaelis and Menten is that, here, the Michaelis constant, K_m, is $(k_2 + k_3)/k_1$, whilst Michaelis and Menten used k_2/k_1, the dissociation constant of the enzyme-substrate complex. In other words, the assumption that k_3 is negligible compared with k_2 is implicit in the expression of Michaelis and Menten, which is now widely adopted. This question of the relative dimensions of these constants is of considerable importance. Calculations of the heat of dissociation of the complex from data on the relation between K_m and temperature [Euler and Laurin, 1920; Dann, 1931] have significance only in as far as k_3 is negligible.

The constant k_3 is itself quite large. Haldane [1930] suggests that it is about 2000 for saccharase at 15°—that is, one molecule of saccharase is capable of hydrolysing 2000 molecules of sucrose per second. Our own estimates from data of Euler and his co-workers suggest a figure about 100.

Knowledge of the relative dimensions of k_2 and k_3 might be gained from a study of the change of rate of hydrolysis starting from the time when the enzyme and substrate were mixed to the time when the maximum rate was reached. As far as we are aware no such study has, as yet, been made and we shall not consider the theoretical aspects of this. What we do propose to

consider is the light thrown on this question by the data on the relation between the rate of hydrolysis and substrate concentration at different concentrations of hydrogen ions.

Michaelis and Davidsohn [1911] found that the relation between rate of hydrolysis of sucrose by saccharase and p_H was almost identical with the relation between the undissociated fraction of a weak acid and p_H. On the basis of this they suggested that saccharase, on the alkaline side of p_H 4·6, behaved as a weak acid and that only the undissociated part combined with sucrose to form a complex capable of forming fructose and glucose. With regard to this picture Haldane says, "It is clear that Michaelis' theory is incomplete, though it has been, and still is, a valuable approximation to the facts." Subsequently Michaelis and Rothstein [1920] found that the rate of hydrolysis changed with p_H in the same manner whatever the concentration of sucrose, in other words K_m was independent of p_H. This caused them to modify the earlier picture to the view that saccharase "verbindet sich mit Saccharose zu einer Verbindung, welche eine Säure ist. Die undissozierten Moleküle dieser Säure zerfallen spontan in monomolekularer Reaktion." As Kuhn [1923] rightly points out the theory does not fit the facts, and he falls back on the explanation that "Die Wasserstoffionen bestimmen die Zerfallsgeschwindigkeit des Saccharase-saccharosekomplexes." Haldane appears to be of the same opinion as Kuhn, for he states that k_3 varies twentyfold with p_H between 4 and 8[1]. As K_m does not alter with p_H he concludes "Unless then we make the quite improbable assumption that both k_1 and k_2 vary proportionally with it, the constancy of K_m shows that k_2 is large compared with k_3." This is difficult to reconcile with Haldane's previous admission that the Michaelis' theory is "a valuable approximation to the facts."

There is another possible explanation of the facts which also throws light on the relative value of k_2 and k_3. The theory embodies that of Michaelis which explains the striking similarity of the relation between rate of hydrolysis and p_H to the relation between the undissociated portion of an acid and p_H—a similarity for which Kuhn and Haldane offer no explanation when they attribute the change of rate to change of k_3 with p_H.

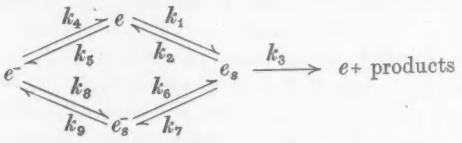
If we assume that on the alkaline side of the optimal p_H the enzyme exists in four forms (not three as the two theories of Michaelis assume)—un-ionised enzyme (e), ionised (e^-), ionised enzyme-substrate complex (e_s^-), and un-

[1] The basis for the statement about the twentyfold change of k_3 appears to be the data of Euler, Josephson and Myrbäck [1924] (quoted on p. 21 by Haldane (1930)) showing a decreased rate of hydrolysis from 100 to 5 when the p_H was increased from 5 to 8. It should be noted that Table IV in Haldane's book inverts the data, giving observed values as calculated, and *vice versa*. Further, it is the calculated values, not the observed values, which are means of the values for five different sugar concentrations at each p_H. The five values for each p_H should have been identical since they are merely the rate at that p_H relative to the value at p_H 5 calculated from the expression

$$\text{Rate} = \frac{kS}{S+k_s} \cdot \frac{H}{H+k_a}.$$

The lack of identity is due to the faulty arithmetic of Euler and his co-workers.

ionised complex (e_s)—the latter alone being capable of giving rise to products at a rate $k_3.e_s$—we can develop an expression for rate of hydrolysis in terms of the concentrations of enzyme (E), substrate (S), and hydrogen ion (H).



This is a long expression and little purpose will be served by presenting it in an unmodified form—the constants are too many for the data available. It becomes more manageable if we assume that the velocity constants of breakdown of e into e^- and H and of their re-combination are the same as the corresponding constants for e_s, the acidic dissociation constant being k_a; and a similar equality for the constants of e_s into e and S, and of e_s^- into e^- and S, the dissociation constant being k_s. So simplified, the expression becomes[1]

$$k_3 e_s = \frac{k_3 E.H}{H+k_a} \cdot \frac{S}{S+\frac{k_2+k_3}{k_1} - \frac{k_2}{k_1(H/k_a+1+S.k_1/k_5+k_2/k_5)}},$$

k_2 is the velocity constant of breakdown of e_s into e and S, and k_5 of e into e^- and H.

In the limit where H is great compared with k_a, as at the optimal p_H, we get the expression of Briggs and Haldane

$$k_3 e_s = \frac{k_3 E.S}{S+(k_2+k_3)/k_1}.$$

As k_3 becomes smaller we approach the expression

$$k_3 e_s = \frac{k_3 E.H.S}{(H+k_a).(S+k_s)}.$$

This is identical with the expression of Euler, Josephson and Myrbäck.

The form of the relation between rate of hydrolysis and substrate concentration should, then, be the same whatever the concentration of H, if k_3 is negligible—a rectangular hyperbola with ordinates proportional to $H/(H + k_a)$ and the Michaelis constant k_s or k_2/k_1 in all cases.

If k_3 is not negligible, but k_5 is small relative to k_2, we get the same expression except that the Michaelis constant is now $(k_2 + k_3)/k_1$ instead of k_s or k_2/k_1. Hence the fact that the Michaelis constant is independent of p_H is, according to our schema, no evidence as to the relative sizes of k_3 and k_2, unless we know that k_5 is not small compared with k_2. The value of k_5 is not known: all we can say is that, since k_a seems to be of the order of 10^{-7}, k_4 is

[1] This expression is obtained by the solution of the four equations

$$k_1 eS + k_4 e_s^- H - e_s(k_2+k_3+k_5) = 0,$$
$$k_5 e_s + k_1 e^- S - e_s^-(k_4 H + k_2) = 0,$$
$$k_5 e + k_2 e_s^- - e^-(k_1 S + k_4 H) = 0,$$
$$e + e^- + e_s + e_s^- = E.$$

about ten million times as big as k_5. If k_2 is of the same order as k_3, about 2000 according to Haldane, then, unless k_4 is of the order of 10^{10}, k_5 will not be great compared with k_2.

It seems worth while considering the effect of p_H on the Michaelis constant assuming that k_5 is not small compared with k_2 but actually as great as that quantity, and k_3 as much as a half of k_2.

The data of Kuhn on the effect of change of p_H on the relation between rate of hydrolysis and concentration of sucrose seem as reliable as any. They seem to lend support to the view that the Michaelis constant is independent of p_H. It is on the basis of these results that Kuhn draws the conclusion about the effect of p_H on the breakdown of the enzyme-substrate complex. The question to be decided is as to whether the results can be explained on the basis of our schema with k_3 half as great as k_2, and the latter equal to k_5. Of course, as we have pointed out above, if k_5 is small compared with k_2 we should expect the Michaelis constant to be independent of p_H, no matter what the size of k_3 relative to k_2. At p_H 4·4–4·6 Kuhn found that the relation between rate of hydrolysis and substrate concentration could be fitted with a rectangular hyperbola with the Michaelis constant 0·040 M. He also concludes that the results at p_H 6·79 are fitted with the constant at the same value. Making our assumptions as to the relative values of k_2, k_3 and k_5 the relation between rate of hydrolysis (A) and substrate concentration at p_H 6·79 should be

$$A = \frac{S}{S + 0{\cdot}04 - 0{\cdot}04/3\,(3 + 37{\cdot}5\,S)},$$

taking H as equal to k_a. The results calculated on the basis of this expression and the observed values, along with those calculated by Kuhn assuming the rate proportional to $S/(S + 0{\cdot}04)$, are given in Table I.

Table I. *Rate of hydrolysis of sucrose by saccharase at* p_H *6·79 (after Kuhn).*

Concentration of sucrose S	Rate of hydrolysis				
		Calculated			
	Observed	I	II	III	IV
0·134 M	18	17	17	78	77
0·066	14	14	14	64	62
0·0415	11·5	11·5	11·5	53	51
0·0252	9	9	9	41	39
0·0128	5·5	5·5	5·5	26	24
0·0073	3·5	3·5	3·5	17	15·5

Calculated I and III from expression $\dfrac{S}{S + 0{\cdot}04 - 0{\cdot}04/3\,(3 + 37{\cdot}5\,S)}$.

Calculated II and IV from $S/(S + 0{\cdot}04)$.

I and II reduced to observed at 0·066 M, III and IV as percentages of rate when S is very great.

The observed rates are clearly given by Kuhn as correct to half a unit, a large quantity for the smaller rates. The rates were determined from curves of hydrolysis against time, by interpolation to find the amount hydrolysed in 10 minutes. The rates are intended as measures of the initial velocity,

before the substrate concentration has changed, and when there are no products of hydrolysis to interfere with the combination of enzyme and substrate. These progress curves are practically straight lines for 20 minutes or more with the higher concentrations of substrate, and hence there is no great difficulty in ascertaining the initial velocity, but with the smaller concentrations the rate falls away more rapidly. An inspection of the progress curves makes one realise that the change in 10 minutes under-estimates the rate of hydrolysis at the lower concentrations, and that in any case the values are certainly not reliable to within less than half a unit. In both sets of calculations the figures have been reduced, so that the calculated rate agrees with the observed when the substrate concentration is 0·066 M and the figures are given as correct to the nearest half-unit. To multiply the observed values and give them correct to 0·5 % as Kuhn does, is unjustifiable. As reduced by the first method the calculated results by both methods are identical.

Hence until we know that k_5 is considerably greater than k_2 we cannot conclude that results such as those of Kuhn, which seem as accurate as any at present, are incompatible with the constant k_3 being comparable in magnitude with k_2, in other words we cannot conclude that the Michaelis constant is equal to the dissociation constant of the enzyme-substrate complex.

There is the possibility that Haldane intends k_3 to be taken as the average rate of breakdown of the complex of enzyme and substrate no matter whether it is ionised or not. In that case, since it appears that only the un-ionised portion of the complex breaks down into products, k_3 or k_h, as we will call it to avoid confusion, is an average of a finite quantity and zero. If

$$H_s = e_s + e_s^- \text{ and } H = e + e^-,$$

the rate of hydrolysis will be given by the expression

$$A = \frac{k_h E.S}{S+(k_2+k_h)/k_1}.$$

Since the rate of hydrolysis varies with p_H it seems that k_h must, and further since $(k_2 + k_h)/k_1$ does not, it appears that k_h must be small compared with k_2, as Haldane argues. But according to our schema $k_h = k_3.e_s/(e_s + e_s^-)$, and from equations on p. 1803 and the expression for e_s when k_5 is small compared with k_2 we find that

$$k_h = k_3 \cdot \frac{S+k_2/k_1}{\{S+(k_2+k_3)/k_1\}(1+k_a/H)-k_3/k_1}.$$

The constant k_h is, then, a function of S as well as of H, except when H is very large, when $k_h = k_3$. Putting this value for k_h in the expression

$$A = \frac{k_h E.S}{S+(k_2+k_h)/k_1}$$

we get back to our expression

$$A = \frac{k_3 E.H.S}{(H+k_a)\{S+(k_2+k_3)/k_1\}}.$$

Thus we see that arguments from an expression using the average rate of breakdown of the enzyme-substrate complex (including ionised as well as un-ionised) can be misleading as to the relative dimensions of k_3 and k_2.

We do not suggest that Haldane uses this particular argument, for we are not sure what precise significance he attaches to k_3, but we cannot admit that his conclusion that k_3 is negligible compared with k_2 is warranted by the facts he produces.

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CXCV. STUDIES ON ANTIGLYOXALASE. II. METHODS OF FOLLOWING GLYOXALASE ACTION.

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Criticism of the manometric method.

In a previous paper [Giršavičius, 1930] an attempt at following glyoxalase action by a manometric method was described. It was thought that the evolution of CO_2 in a bicarbonate-CO_2 buffer system would provide a convenient means of following the production of lactic or mandelic acid under the action of glyoxalase. It was soon found, however, that a gas evolution, indistinguishable from that caused by the investigated reaction, takes place when phenylglyoxal and pancreatic extract are brought together. Further investigation showed that this reaction is not abolished by first boiling the pancreatic extract, and that boiled extracts of testis and of yeast show the same phenomenon, whilst boiled liver and kidney extracts do not. As the reaction is abolished after treating the tissue extract with nitrous acid, or after precipitation with phosphotungstic acid, it was considered to be due to amino-compounds. It was then shown that, although monoamino-acids do not cause more than a slight gas evolution with phenylglyoxal, diamino-acids cause a rapid gas evolution; the conclusion was drawn that diamino-acids or related compounds, or polypeptides containing a high proportion of diamino-acids, are responsible for the reaction in question. Later work has shown, however, that proteins and peptones also cause a gas evolution in the Barcroft manometer, and that this reaction is apparently characteristic for all protein derivatives, although with monoamino-acids it is comparatively slow.

The difference between liver and kidney extracts on the one hand and extracts of pancreas, yeast and testis on the other hand seems to be due to differences in the amount of non-heat-coagulable N they contain, rather than to the proportion of diamino-acids entering into their composition.

Exp. 1. In the following experiment the effects of liver and of pancreatin on phenyl- and on methyl-glyoxal were compared. 0·084 *M* solutions of phenyl- and methyl-glyoxal were prepared, making use of the modified Friedemann method described in this paper.

0·5 cc. of these was tipped into the following solutions:

(1) with liver extract,

(*a*) 1 cc. liver extract, 1 cc. water, 0·7 cc. sodium bicarbonate solution, and, in the small cup, 0·5 cc. phenylglyoxal.

(*b*) like (*a*), but methylglyoxal instead of phenylglyoxal;

(2) with pancreatin solution (2 % pancreatin B.D.H.),

(*a*) 1 cc. pancreatin solution instead of liver extract, otherwise as (1, *a*),

(*b*) like (*a*), but with methylglyoxal.

All solutions had a p_H of 7·6. The results are given in mm.^{3} CO_2.

Minutes	(1 *a*)	(1 *b*)	(2 *a*)	(2 *b*)
4	110	60	37	14
8	154	78	47	20
15	198	98	55	29
21	225	114	—	34

Both with liver and with pancreatin the reaction proceeds at almost exactly twice the rate with phenylglyoxal as with methylglyoxal. This seems to show that not only the reaction with pancreatin, but also the reaction with liver observed under these conditions, is largely the protein reaction, rather than the reaction due to glyoxalase.

It is clear from the foregoing results that the manometric method cannot be used for following glyoxalase action. In no case is it possible to be certain how much of the reaction observed is due to glyoxalase and how much to the proteins and protein derivatives present in the solution.

In a recent paper, Lohmann [1931] has used the manometric method for a study of the co-enzyme of glyoxalase. From his results he concludes that glyoxalase preparations inactivated by dialysis, and, to a somewhat lesser extent, fresh glyoxalase preparations, are activated by the addition of a "co-enzyme" preparation. This he obtained by treating a trichloroacetic acid extract of yeast with lead acetate and mercuric nitrate. Unfortunately he gives no control figures for the effect of this preparation itself on methylglyoxal; it is therefore impossible to judge from the results he reports whether he was dealing with a true co-enzyme, or whether the activation observed was really a summation of two reactions (as when pancreatin is added to liver extract).

Since the manometric method had thus proved itself unsuitable as a means of investigating glyoxalase action, a different method had to be sought. Two ways of attacking this problem presented themselves. One could either, as Dakin and Dudley (and in some of their work Kuhn and Heckscher [1926, 1, 2] had done, estimate polarimetrically the mandelic acid produced from phenylglyoxal; or, like Kuhn and Heckscher in the main part of their work, Ariyama [1928] and Sakuma [1930], follow, by some suitable method, the disappearance of the glyoxal. As the estimation of the acid is rather laborious, the second way was chosen. It was clear, of course, that the reaction with proteins would have to be taken into account, and might interfere considerably; but whether this

effect would be large enough to make this method as unsuitable as the manometric method, could not be decided *a priori*—especially as such a method had already been used by the above authors. The first problem was to find a satisfactory way of estimating glyoxals.

Modification of Friedemann's method for use as a micro-method.

Two methods were available for estimating glyoxals; the iodimetric method of Kuhn and Heckscher, and the colorimetric method of Ariyama[1]. Of these the former seemed less satisfactory, as it is not quite specific, but mainly because of the two-hour periods required before each titration. Ariyama's method has the disadvantage that it can only be used within narrow limits of concentration with one standard. A rapid titrimetric method seemed preferable, if it could be designed. Friedemann [1927] had shown that glyoxals are quantitatively oxidised by hydrogen peroxide in alkaline solution giving two equivalents of acid for each mol. of glyoxal. Phenylglyoxal, for instance, is oxidised to an equimolar mixture of benzoic and formic acids. Friedemann based on these facts a method for estimating various glyoxals by titrating the acids produced. This method has not been applied in actual practice, except by Ariyama, who used it to standardise his methylglyoxal solutions. It seemed probable that if it could be modified into a micro-method of sufficient accuracy and sensitivity, Friedemann's method would have the advantage of being very rapid and simple to carry out.

For accurate acidimetric titration of dilute solutions it is necessary to determine the end-point with reference to a standard. It is also necessary to control the indicator concentration, which can be done in two ways: either the standard is diluted parallel with the titrated fluid, or else more indicator is added during the titration, so as to keep its concentration constant. The first method is preferable, as coloured solutions can be titrated fairly accurately by its use. It is convenient to carry out the titrations in front of a box with a plate glass front illuminated from inside.

The following solutions are placed in five boiling-tubes: *A*, *B*, *s*, *1*, *2*.

A, CO_2-free water, containing 1 cc. phenolphthalein per 10 cc., and adjusted to a rose-pink colour with a few drops of *N*/50 NaOH.

B, 20 vol. H_2O_2 diluted 1 : 5, containing 1 cc. phenolphthalein per 10 cc., and adjusted to the same colour as *A*. The colour of this solution gradually fades on standing, owing to destruction of the indicator; from time to time a few drops of phenolphthalein have to be added, to readjust the colour.

[1] Recently Barrenscheen and Dreguss [1931] have published another method for the estimation of glyoxals. They claim it to be very specific, and, judging by their figures, it seems to be more sensitive and perhaps more accurate than the method described in this paper. On the other hand, it is far more cumbersome. Where specificity, or great accuracy at high dilution, are required, the method of Barrenscheen and Dreguss (or Ariyama's method) will be preferable. Where these considerations are less important, the method here described will probably be sufficiently accurate and at the same time simpler and more rapid.

s, *1*, *2*, receive 10 cc. each of the solution to be titrated, and 1 cc. phenolphthalein. *s*, which serves as standard for the titration, and the two other tubes, are then adjusted to the same colour as *A* and *B*.

1 cc. *A* is now added to *s*, and 1 cc. *B* to *1* and *2*. If the solution contains any methyl- or phenyl-glyoxal the colour in *1* and *2* will fade or disappear immediately on adding the hydrogen peroxide. *1* is then titrated with *N*/50 NaOH to a permanent red colour. The same amount of NaOH is added to *2* (it is convenient to have two burettes attached to the bottle containing the NaOH); to *s* is added the same volume of CO_2-free water. After standing about a minute, tubes *1* and *2* are adjusted to the colour of *s* with *N*/50 HCl, again with addition of CO_2-free water to *s*. It is clear that the sharpness of the end point will depend upon the absence of substances exerting a buffer action within the p_H range of the indicator used. With phenolphthalein as indicator phosphate has little buffering power. The buffer action of borate can be shifted into a lower p_H range by adding a glycerol solution to tubes *s*, *1*, *2*. On the other hand, carbonates present in the original solution, and CO_2 absorbed from the atmosphere, definitely diminish the sharpness of the readings. The figures of Table I may serve to illustrate the accuracy obtainable.

Table I.

Phenylglyoxal cc.	NaOH (found) cc.	NaOH (average) cc.	NaOH (calculated) cc.
3	6·08	6·06	—
3	6·02		
3	6·09		
1	2·03	2·01	2·02
1	1·99		
0·5	1·00	1·02	1·01
0·5	1·04		
0·5	1·05		
0·5	1·01		
5	2·10	2·13	—
5	2·16		
5	2·16		
5	2·13		
5	2·10		
2	0·84	0·86	0·85
2	0·87		
2	0·88		
2	0·84		
1	0·44	(0·43)	0·42
1	0·43		
1	0·42		
1	(0·61)		
1	0·42		

As 1 mol. of the various glyoxals gives rise to 2 equivalents of acid, 1 equivalent of NaOH corresponds to 36 g. methylglyoxal or 67 g. phenylglyoxal. 1 cc. *N*/50 NaOH corresponds to 0·72 mg. methylglyoxal, or 1·34 mg. phenylglyoxal.

For the estimation of phenylglyoxal in solutions containing tissue extracts, 25 cc. are pipetted into 50 cc. volumetric flasks, containing 2 cc. 10 % trichloroacetic acid. After standing for some minutes the liquids are made up

to the mark and filtered through dry pleated filters (Dreverhoff's "Rapid Filtrierpapier" is very convenient) into small dry flasks. Samples of 10 cc. are then pipetted out for analysis, and treated as described above. Separate experiments have shown that the concentration of phenylglyoxal is not altered by precipitating protein in the solution and filtering.

The main defect of the present method lies in its lack of specificity. Acetaldehyde does not interfere. Although it is gradually attacked under the conditions of this method, the reaction is so slow that even high concentrations of acetaldehyde introduce no appreciable error. On the other hand pyruvic acid and dihydroxyacetone behave exactly like methyl- and phenyl-glyoxal, and it is possible that this method may be of some use in their estimation, where confusion is out of the question. The possible confusion with dihydroxyacetone, and particularly with pyruvic acid, of course diminishes considerably the usefulness of the method for the estimation of glyoxals.

Investigation of glyoxalase action by following glyoxal disappearance.

In the work now to be described the modified Friedemann method has been made use of for following the disappearance of phenylglyoxal under the action of glyoxalase and under various other conditions. The following typical experiment on the action of pancreas will serve to illustrate the method as used in practice.

Exp. 2.

Solution (1): 20 cc. liver extract, 20 cc. phosphate buffer, $p_H = 7{\cdot}6$, 20 cc. water.
Solution (2): like solution (1), but instead of water 20 cc. pancreatic extract.

These solutions were incubated overnight at 38°[1]. 25 cc. phenylglyoxal solution were then added to each and samples were taken for analysis immediately and after 40 and 80 minutes. The following are the results, the figures being the differences between the initial and final titrations (cc. $N/50$ NaOH):

Initial phenylglyoxal = 6·49 cc. NaOH.

Minutes	(1)		(2)	
40	0·20	0·29	0·54	0·68
80	—	0·67	0·84	0·86

It will be seen that in the present experiment not only does the addition of pancreas not cause any inhibition, but in its presence actually more phenylglyoxal disappears than without it. The following experiment explains this phenomenon.

Exp. 3.

Solution (1): 50 cc. liver extract, 15 cc. buffer, 60 cc. water.
Solution (2): 50 cc. water, 15 cc. buffer, 60 cc. 0·4 % pancreatin B.D.H.
Solution (3): 50 cc. liver extract, 15 cc. buffer, 60 cc. pancreatin.

The solutions were incubated for 5½ hours. 50 cc. of each were then pipetted into flasks containing 20 cc. buffer and 10 cc. phenylglyoxal solution. Samples were taken immediately and after 1 hour.

[1] In this as in all following experiments toluene was added as an antiseptic.

Initial phenylglyoxal = 4·27 cc. $N/50$ NaOH.

Phenylglyoxal destroyed after 1 hour equals (in cc. $N/50$ NaOH):

(1)		(2)		(3)	
0·95	0·93	0·19	0·18	1·17	1·10

It will be seen that the destruction of phenylglyoxal by liver and by pancreatin alone have been exactly summed, when both were incubated together; there was neither inhibition nor acceleration. It is, however, possible to demonstrate antiglyoxalase action, as the following experiments with a different sample of pancreatin show.

Exp. 4.

Solution (1): 50 cc. liver, 20 cc. water, 20 cc. buffer.

Solution (2): 50 cc. water, 20 cc. pancreatin solution, 20 cc. buffer.

Solution (3): 50 cc. liver, 20 cc. pancreatin solution, 20 cc. buffer.

After incubation overnight 30 cc. of these solutions were pipetted into flasks containing 20 cc. buffer and 10 cc. phenylglyoxal solution. Samples were taken immediately, after 35 minutes and after 1 hour and 30 minutes.

Initial phenylglyoxal = 2·58 cc. $N/50$ NaOH.

Minutes	(1)	(2)	(3)	(3)–(2)
35	0·52	0·06	0·39	0·33
150	1·50	0·33	0·76	0·43

In the fourth column the figures obtained by subtracting the phenylglyoxal destruction by pancreatin alone from the phenylglyoxal destruction by liver *plus* pancreatin, are tabulated. Whether this correction is applied or not, the figures show a considerable inhibition.

Exp. 5. In this experiment a larger amount of pancreatin was used. The solutions were treated as in the preceding experiment; the results alone will therefore be given.

Initial phenylglyoxal = 6·92 cc. $N/50$ NaOH.

The amounts of phenylglyoxal destroyed after 1 hour equal:

(1) Liver alone	(2) Pancreatin alone	(3) Liver *plus* pancreatin	
2·26	2·04	2·36	2·33

If the destruction of phenylglyoxal by pancreatin is quantitatively summed in (3) with the residual activity of the liver extract, almost complete inhibition is shown by these figures. This experiment, however, also shows clearly the reason why this method was finally abandoned as unsuitable for the study of glyoxalase action. The destruction of phenylglyoxal by pancreas alone is far too large to be ignored, in fact the correction that has to be applied for it is in many experiments considerably larger than the effect investigated—the inhibition by antiglyoxalase. It is impossible, under these conditions, to obtain reliable results.

Moreover, other experiments have shown that, as with the gas evolution measured manometrically, the destruction of phenylglyoxal by pancreatin and

pancreatic extracts is only a special case of a reaction with proteins and their derivatives. It is clear therefore that not only has the destruction of phenylglyoxal by liver treated with pancreas to be corrected for the destruction by pancreas alone, but, which is even more important, a considerable and not readily assessable proportion of the phenylglyoxal destruction by liver extracts (and those of other tissues) is due, not to glyoxalase, but to a reaction with the tissue proteins. This makes it impossible to interpret with any certainty the results obtained. It can be concluded from these experiments that it is impossible to follow glyoxalase action in more than the roughest qualitative manner by following the disappearance of the substrate. Glyoxalase can only be studied reliably by estimating the lactic or mandelic acid produced.

Much of the work so far done on glyoxalase (Kuhn and Heckscher, Ariyama, Sakuma) has depended on the estimation of glyoxals by various methods. The results obtained in this way have to be accepted with some care, as they cannot be relied upon to represent actual glyoxalase action. Only the work carried out with Dakin and Dudley's technique is free from this particular objection.

The rather irregular kinetics found by Kuhn and Heckscher [1926, 1] in their study of methylglyoxalase, and the variable results these authors obtained, for instance on dialysing their extracts, are probably partly to be explained by the phenomena described above. The p_H-activity curves obtained by Kuhn and Heckscher (fall in activity beyond p_H 8), as well as the diminishing activity of their extracts on standing, show that their results are at any rate partly, perhaps mainly, due to glyoxalase action. Non-enzymic methylglyoxal destruction must, however, have gone on simultaneously, so that their results cannot be interpreted with certainty. Kuhn and Heckscher [1926, 2] also noted the destruction of methylglyoxal by boiled tissue extracts, causing an apparent acceleration of glyoxalase, but did not follow up this observation. Sakuma's work calls for similar comments. This author observed the destruction of methylglyoxal by proteins and various amino-compounds. According to his observations the reaction is a rather complex one; simultaneously with the disappearance of methylglyoxal there is an equivalent production of NH_3 and CO_2. This probably explains the gas evolution observed when using the manometric method, which in the previous paper of this series was tentatively, and apparently erroneously, ascribed to the action of some acid compound of phenylglyoxal and an amino-acid. Whilst Sakuma has studied this reaction in some detail, he has failed to see that it greatly diminishes the value of his work on the distribution of glyoxalase, and on the "co-enzyme" of glyoxalase. That such a co-enzyme is active in boiled tissue extracts, like those of Sakuma, is possible. In view of the work of the Neuberg school it is even quite probable. His results do not, however, prove the existence of such a co-enzyme, especially as he gives no control figures for the destruction of methylglyoxal by his "co-enzyme" solutions alone. In his Table V, for instance, boiled extracts of liver, muscle, and kidney are added to a liver and to a muscle extract. The percentage activations obtained differ widely, being far greater with the weakly active muscle extract. If, however, the differences, in other words the excess

activities when boiled extracts are added, are compared instead, it is found that each of the boiled extracts produces practically the same increase in activity, whether it is added to the liver or to the muscle extract. Again, if the results of his Table VI, where increasing amounts of boiled liver juice are added to liver extracts, dialysed for various periods of time, are plotted, it will be seen that the points fall practically on parallel straight lines, differing only in their point of origin, according to the activity of the liver extracts themselves. These results are more consistent with a summation of activities, as in Exp. 3 and others of the present paper, than with a co-enzyme activation. At any rate the question is still open. The inhibition by phosphate and the exceedingly interesting acceleration by sugars, which Sakuma reports, are more likely to be properties of glyoxalase itself. But all these require re-investigation.

It will be seen from the present paper, that only results obtained by estimating the acid produced can give a true picture of glyoxalase action. So far the polarimetric estimation of mandelic acid is the only method available for this purpose. This method, as used by Dakin and Dudley, is rather cumbersome. Some improvements have therefore been introduced, and will be described in a subsequent paper, together with the results obtained.

Summary.

1. Manometric methods, based on Warburg's glycolytic technique, are unsuitable for the study of glyoxalase as glyoxals cause a gas evolution with proteins and their derivatives.

2. A volumetric method for the estimation of glyoxals is described. This method makes use of the fact, discovered by Friedemann, that glyoxals are oxidised to two equivalents of acid by H_2O_2 in alkaline solution.

3. By means of this method it is shown that, owing to its relatively high rate, the reaction between glyoxals and proteins cannot be ignored, or even adequately corrected for. This makes results obtained by following glyoxal disappearance very unreliable as expressions of glyoxalase action. In this connection some of the work of other authors, who used similar methods, is discussed critically.

4. It follows that glyoxalase action can only be reliably investigated by estimating the acid produced.

I would like to express my thanks to Sir F. G. Hopkins and to Prof. J. B. S. Haldane for their interest and kind encouragement.

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CXCVI. FERMENTABLE SUGAR IN NORMAL URINE.

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Introduction.

The problem of true sugar in blood has now received a satisfactory solution. There may be still a little doubt as to whether a few mg. may not be a true sugar other than glucose, but within that limitation the amount can be estimated readily and accurately. It is not so with the presence of true sugar in urine. The urine of all normal persons contains carbohydrate reducing material, but to what extent this consists of glucose has been a matter of controversy. The older work has already been reviewed from time to time [Benedict, Osterberg and Neuwirth, 1918; Folin and Berglund, 1922, 2; Greenwald, Gross and Samet, 1924]. As regards more recent work, there have been no fundamentally new methods of analytical approach to the problem. Observers have still to depend on a yeast fermentation process, or on the formation of osazones.

The danger of contamination by bacteria, in using yeast for the estimation of glucose has been well recognised. Excluding this error, Lund and Wolf [1926] failed to observe CO_2 formation as a result of the incubation of pure yeasts and urine, and Patterson [1926] by incubating ordinary yeast and urine in presence of toluene noted no change in reducing power. Hiller, Linder and Van Slyke [1925] by using larger amounts of yeast shortened the time required for the removal of glucose from aqueous solutions and blood. Eagle [1927] applied the method to urine, and reported the entire absence of fermentable sugar, either fasting or after ordinary meals. His figures have received a correction from Van Slyke and Hawkins [1929], but even so the amount must be under 10 mg./100 cc. urine. On the other hand Greenwald, Gross and Maguire [1927], using Eagle's technique found fermentable sugar after meals but none after glucose in some subjects, and concluded that normal urines contain reducing substances other than glucose that are removed by treatment with yeast. In a series of after-breakfast urines Van Slyke and Hawkins have noted significant amounts of fermentable sugar. Peterson and West [1929] have also observed in 50 normal urines daily excretion of fermentable reducing substances in amounts varying widely up to 400 mg.

[1] Assisted by a grant from the Banting Research Foundation, University of Toronto.

The use of phenylhydrazine has led to similar contradictory evidence, Patterson [1926], Geelmuyden [1915], Höst [1923], have all failed to prepare *d*-phenylglucosazone from normal urines, contradicting the earlier positive claims. More recently however, Hassan [1928], removing interfering substances by charcoal, prepared typical glucosazone crystals from urine 1–2 hours after an ordinary meal in 20–30 % of 700 Egyptian students. After a 12 hours fast the occurrence of glucose was reduced to 7 %. Glucosazone may be valueless as a specific indication of glucose in urine. Quite apart from the possible presence of fructose or mannose, Greenwald, Gross and Maguire [1927] claimed the isolation of *d*-phenylglucosazone from normal urine which contained only non-fermentable carbohydrate reducing material, and drew attention to the possible formation of compounds of this type from sucrose.

A very complete examination of the problem was made by Malmros [1928] who, from evidence of osazone formation combined with yeast fermentation concluded that "even though it has not been possible to produce a chemical-analytically perfectly valid proof, the investigations still indicate that glucose occurs in normal urine."

Other investigators have approached the problem from the physiological standpoint. Thus Folin and Berglund [1922, 2] cited the evidence of blood-sugar studies after the ingestion of large amounts of pure glucose, together with the non-occurrence of any rise in the carbohydrate reducing material in the urine, against the possibility of the occurrence of glucose in normal urine. The experimental work and its value was severely criticised by Benedict and Osterberg [1923], who together with Neuwirth [1918] had put forward a view involving a continuous secretion of glucose into the urine.

Both from the clinical and the laboratory side of medicine during the last decade, there has come an increasing recognition of two types of normal people who consistently show glycosuria. The first, renal glycosuriacs [Goto, 1918; Marsh, 1921], possess a sugar threshold below the normal value and both in the fasting condition and after meals show sugar in the urine. It is recognised too that the threshold for sugar can be found at any level of the blood-sugar [Goto and Kuno, 1921; Gray, 1923; John, 1923], so that individuals will occur who show absence of glucose from fasting urine, but its presence after glucose ingestion or after meals, though the blood-sugar curve may not reach the usual normal renal threshold. The second type have been called "cyclic hyperglycaemics" [Holst, 1925; Faber, 1926] or "lag type people" [MacLean, 1924; Linder, Hiller and Van Slyke, 1924]. In them the blood-sugar rises transiently above the usual value for the renal threshold after glucose ingestion, or after meals, with resulting glycosuria.

In discussing the presence of glucose in normal urine it is usually assumed that these types are not very common, and in experiments on a small series of subjects such types are excluded by direct observation. The real problem of glucose in normal urine lies in determining whether individuals showing no clinical tests for sugar, and presumably possessing glucose tolerance curves

of the "ideal" type [MacLean, 1924; Linder, Hiller and Van Slyke, 1924], actually excrete small amounts of glucose. In view of the contradictory evidence which we have summarised, four possibilities present themselves.

(1) A difference between the fasting condition and after meals.

(2) The occurrence of subjects with thresholds for sugar lower than normal but not low enough to allow the escape of sufficient sugar to be detected clinically.

(3) The occurrence of subjects responding to glucose or carbohydrate ingestion by a hyperglycaemia above the normal threshold, but not showing sufficient glycosuria to be detected clinically.

(4) The urinary sugar reported as fermentable, or as giving *d*-phenylglucosazone, is not glucose.

Experimental.

Analytical methods.

The analytical procedures for urine are as follows:

A. Preliminary dilution. Normal urine is diluted 5 or 10 times. Urines showing positive Benedict sugar tests are diluted 20, 50 or 100 times. Very dilute urines are diluted only 1:1 or 1:2. The dilutions are arranged so that the final titration lies between the values 0·3 and 1·5 cc. of 0·005 *N* thiosulphate solution. It is to be noted that the final dilution is always twice the preliminary dilution.

B. Total "sugar." 5 cc. of the diluted urine, 5 cc. of 0·04 *N* H_2SO_4 are mixed, 0·3 g. Lloyd's reagent added; the mixture shaken and centrifuged. 2 cc. of the supernatant liquid are mixed with 2 cc. of the Shaffer-Hartmann copper solution and used as described for pure glucose under the heading "Standardisation of the copper solution."

C. Non-fermentable "sugar." 1 cc. of a 25 % suspension of washed yeast is centrifuged, the fluid adherent to the centrifuge tube is removed by filter-paper, and to the cells are added 5 cc. of the diluted urine. The cells and liquid are mixed and incubated 8 mins. at 38°, the mixture being agitated two or three times. 5 cc. 0·04 *N* H_2SO_4 are added, mixed and then 0·3 g. Lloyd's reagent, added. After shaking and centrifuging 2 cc. of the supernatant liquid are used for the determination of non-fermentable "sugar" as in total sugar.

D. Blank determination. 5 cc. water are mixed with 5 cc. 0·04 *N* H_2SO_4; 0·3 g. Lloyd's reagent added, and the mixture is shaken and centrifuged. 2 cc. supernatant liquid are used, as in determinations B or C. A blank determination must be carried out with each batch of reductions.

E. Fermentable sugar. The difference B–C properly corrected for the blank represents fermentable sugar.

Recovery of added sugar from urine. Added glucose (100 mg./100 cc.) is recovered either directly or by fermentation from all human urines so far examined. Fructose, however, in concentrations greater than 25 mg./100 cc. is not recovered quantitatively from urine by yeast under our conditions. In lower concentrations the recovery is quantitative.

In presence of a mixture of phenols representing 30 mg./100 cc. phenol the recovery of glucose is similar to that from urine.

Preparation of a modified Shaffer-Hartman micro-reagent[1].

Copper sulphate	5·0 g.
Tartaric acid	7·5 „
Sodium carbonate anhyd.	40·0 „
Potassium iodate	0·7 „
Potassium oxalate	18·4 „
Water to	1000 cc.

[1] The details of this solution and its preparation were kindly supplied to us by Prof. P. A. Shaffer. The notes and criticisms represent the experience of this laboratory after 4 years' use.

The copper sulphate and tartaric acid are dissolved in about 150 cc. of water. The sodium carbonate is dissolved in about 500 cc. of water and the iodate and oxalate in about 150 cc. of water. The solutions are all cooled to 25° and the sulphate-tartaric acid solution added slowly to the carbonate solution with gentle agitation. The temperature is not allowed to rise above 27°. The iodate-oxalate solution is then added and the whole transferred quantitatively to a volumetric flask, and brought to 1000 cc.

A separate solution of 1 % KI.

It is advisable to allow the copper solution to stand 3 weeks before use. This allows a slight amount of precipitate to settle. The solution, once made, does not alter in its oxidising power towards glucose. During the first 3 weeks the oxidising power towards the non-glucose reducing substances in blood alters slightly but after this interval it remains constant. We have used solutions over a year old, without finding any alterations in the values obtained against pure glucose, though the value of the blank increases slightly.

Standardisation of the copper solution. Solutions of pure glucose of 20, 10, 5, 2·5 and 1 mg./100 cc. are prepared.

2 cc. of the copper solution and 2 cc. of the sugar solution are mixed and heated exactly 10 mins. in a rapidly boiling water-bath in a $6'' \times \frac{3}{4}''$ tube, stoppered with a loose plug of cotton wool. The tube is cooled to 30° and 2 cc. of the KI solution are added. The I_2 is liberated by 2 cc. of N H_2SO_4 with shaking and determined by titration with 0·005 N thiosulphate solution.

We have used 1·0 % starch solution in cold saturated solution of phenol red as indicator. A blank determination with water is also carried out with each batch of reductions. The blank value is subtracted from the sugar value. The difference between duplicate titrations should not be greater than 0·02 cc. of 0·005 N thiosulphate. If desired greater or less amounts of sugar solution can be used with a corresponding increase or decrease in the volume of the reagents.

Sensitivity of the reagent. In water solution the reagents will estimate 0·1 mg./100 cc. of glucose using triplicate determinations.

Stability of reduction products. The solution besides being much more sensitive to glucose than the original Shaffer-Hartman is also superior in the stability of the cuprous compounds, formed in the reduction. The values for glucose over a range of 0·2–10·0 mg. per 100 cc. are the same in an atmosphere of N_2 as under the described ordinary conditions.

Values with blood-filtrates and urines. The method can be used with blood-filtrates prepared by the Folin-Wu method, the Somogyi precipitation with $ZnSO_4$ and NaOH, and the Herbert and Bourne method with 3 % Na_2SO_4 and tungstic acid. 0·3 cc. finger blood precipitated according to Folin-Wu was used for the figures reported in this paper.

Whilst the behaviour of the solution to glucose is constant, we have found variable results when the non-sugar fermentable material of blood is included in the determination [*cf.* Harding and Van Nostrand, 1930]. The cause of this variation we now suspect to be the bicarbonate content of our copper solution. Somogyi [1926] noted the effect of variations in the carbonate-bicarbonate ratio on the glucose values of the Shaffer-Hartman solution and as a result devised a modification containing both carbonate and bicarbonate. A similar variation evidently affects the non-sugar reducing material, as may be shown by passing into the new Shaffer-Hartman solution a stream of CO_2 and determining the values for fermentable and non-fermentable sugar in a Folin-Wu filtrate. Both are lowered, the glucose value to a greater degree than the non-fermentable value.

The values for the non-fermentable sugar in urine have varied very little from one copper solution to another prepared in the usual standard way.

Values with sugars other than glucose. The solution can be used for fructose, galactose and lactose, in water solution, or added to blood-filtrates or urine. Lactates show no reducing values nor iodine absorption values to our reagents.

Use of Lloyd's reagent for removal of interfering substances in urine previous to "sugar" determination. The necessary removal of uric acid, creatinine, *etc.* from urine previously to the application of the modified Shaffer-Hartman reagent may be effected by the original method suggested by Folin and Berglund [1922, 1] of Lloyd's reagent and H_2SO_4. It seems unnecessary to use the modifications of Folin and Svedberg [1926], or Hamilton [1928] under our conditions. The use of Lloyd's reagent, however, does necessitate the determination of a new blank value, in which water previously treated with H_2SO_4 and Lloyd's reagent under the same conditions as urine, replaces water alone.

Removal of fermentable sugar from urine by yeast. We have used Fleischman's yeast washed with water according to the directions of Somogyi [1927]. The stock washed yeast was weighed out when required to make a 25 % suspension by weight with distilled water. We have not found necessary the removal of interfering substances by Lloyd's reagent before the application of the yeast recommended by Eagle [1927].

Van Slyke and Hawkins [1929] have recently stated that yeast applied to urine not only removes fermentable sugar but in addition a variable percentage of the non-fermentable material remaining after treatment with Lloyd's reagent. This results in a correction being necessary in their technique for the determination of fermentable and non-fermentable "sugar" in urine.

We have examined our own technique to see if any such absorption of non-fermentable "sugar" is noticeable. Samples of fasting normal human urine, on which the total "sugar" was first determined, were treated with 3 successive portions of yeast as described in C and the residual non-fermentable sugar determined after each yeast treatment. The results are shown in Table I. The yeast treatments show many times that the urine has gained a little in "sugar" instead of suffering a loss as would be expected were any appreciable amount of non-fermentable yeast-absorbable substance present. The changes in either direction are small, and are of the same magnitude as the thiosulphate titration error. Thus it becomes evident that under our analytical conditions no yeast correction is necessary. Whether the difference between our results and those of Van Slyke and Hawkins lies in the higher dilutions of the urine used by us, or in the use of a copper solution as the reagent for "sugar" determination rather than a ferricyanide reagent, we do not know.

A minor but by no means negligible error can be introduced by yeast in either urines or blood-filtrates by insufficient centrifuging. A very small number of yeast cells, remaining in the supernatant liquid and thus being pipetted into the modified Shaffer-Hartman reagent, causes a marked increase in reduction. In our most accurate work we found it necessary to centrifuge the supernatant liquid a second time.

Table I. *Showing effect of successive treatments with washed yeast on total "sugar" in normal fasting human urine.*

2 cc. of 1:10 diluted urine according to B or C.

Specimen No.	Total "sugar" initial	After yeast treatment 1	2	3	Loss or gain after each yeast treatment 1	2	3
		cc. 0·005 *N* thiosulphate			cc. 0·005 *N* thiosulphate		
1	1·67	1·65	1·62	1·59	−0·02	−0·03	−0·03
2	1·29	1·25	1·27	1·28	−0·04	+0·02	+0·01
3	1·06	1·08	1·06	1·07	+0·02	−0·02	+0·01
4	0·68	0·70	0·72	0·74	+0·02	+0·02	+0·02
5	0·67	0·66	0·64	0·67	−0·01	−0·02	−0·03
6	0·64	0·64	0·64	0·64	±0·00	±0·00	±0·00
7	0·35	0·37	0·35	0·38	+0·02	−0·02	+0·03
8	0·32	0·35	0·35	0·35	+0·03	±0·00	±0·00
	Total change after yeast treatments				+0·02	−0·05	+0·07

Fasting and after glucose.

The subjects represented in Table II were medical students. No food was taken after the meal of the previous evening, the night urine was discarded at 7 a.m., 200 cc. of water were taken, and the student arrived at the laboratory a little before 9 a.m. The subject rested quietly for 15 mins. The 7 to 9 a.m. urine specimen was voided, the initial blood sample taken, and 50 g. glucose in 200 cc. water were given. Finger blood was taken every 15 mins. during the first hour and at the 30 min. interval in the second hour. After 9 a.m. the subjects performed their ordinary laboratory work. An extra 200 cc. of water

was allowed if the 7–9 a.m. specimen had been scanty. The second urine specimen was collected at 11 a.m. All analyses were performed immediately. The blood-sugar curves are shown in Figs. 1 and 2. Table II shows the difference between the total sugar and the non-fermentable "sugar" in cc. 0·005 *N* thiosulphate solution for 2 cc. of urine-filtrate, and the amount of fermentable sugar in the 2-hour sample as glucose. The small amount of thiosulphate—not more than 0·03 cc.—representing the fermentable fraction in the 7–9 a.m. specimen is in the same range of error as two duplicate determinations. The average 0·01 cc. is the same both in the fasting urine and after glucose. Subjects 5 and 9

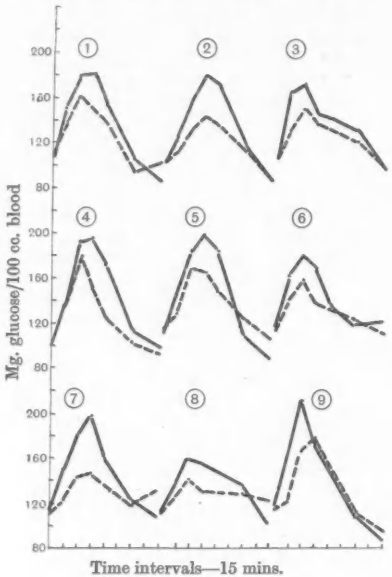

Fig. 1.

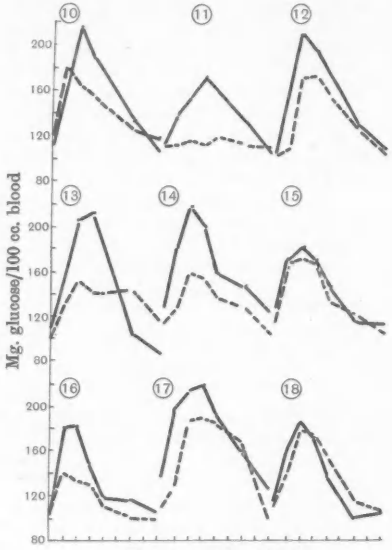

Fig. 2.

Figs. 1 and 2. Showing morning and afternoon tolerance curves after 50 g. glucose.
Morning curves ------- Afternoon curves ———

after glucose show a doubtful result. The amount is so small that the figures can at present have no significance. This does not mean to say that absolutely no glucose escapes into the urine. There may be amounts up to 2 mg./100 cc. present which is the smallest detectable by our method. Peters and Van Slyke [1931] in their recent book, summing up the available evidence, give the actual glucose content of urine as 1 mg./100 cc.

Subject 2 however, has undoubtedly allowed a few mg. of glucose to escape into the urine after glucose ingestion. His renal threshold is a little low, as the maximal blood-sugar observed after glucose ingestion was 142 mg./100 cc. (see Fig. 1). He represents however, only 1 subject out of 18

and it is evident that the occurrence of such variations is not very common. The usual "normal" possesses the usual threshold value for glucose. On the other hand relying on the osazone formation Malmros [1928] is inclined to think that 40 % represents an average rate of occurrence of physiological glycosuria. This is a percentage very much higher than ours. The difference may perhaps be racial, but may be due to our more careful exclusion of any subjects likely to be considered as mild renal glycosuriacs.

Table II.

	7–9 a.m. Fasting		9–11 a.m. 50 g. glucose at 9 a.m.	
No.	0·005 *N* thiosulphate for 2 cc. urine filtrate cc.	Fermentable sugar per 2 hr. mg.	0·005 *N* thiosulphate cc.	Fermentable sugar per 2 hr. mg.
1	−0·005	0·3	−0·01	0·5
2	+0·01	0·3	+0·09	5·1*
3	±0·00	0·0	−0·03	−3·3
4	+0·02	0·8	+0·02	2·8
5	+0·03	2·0	+0·05	2·8
6	+0·03	2·0	+0·02	1·4
7	+0·03	0·0	+0·02	1·2
8	+0·01	0·0	±0·00	0·0
9	+0·03	1·5	+0·04	3·2
10	±0·00	0·0	−0·01	0·0
11	±0·00	0·0	−0·01	0·7
12	±0·00	0·0	−0·02	1·0
13	±0·00	0·0	±0·00	0·0
14	+0·01	1·1	−0·02	−1·5
15	+0·01	0·4	−0·01	−1·8
16	±0·00	0·0	±0·00	0·0
17	−0·02	−0·5	+0·01	0·7
18	+0·01	0·0	+0·03	2·0
Average	+0·01	0·4	+0·01	0·4

* Excluded from average.

In view of the high peaks for blood-sugar shown in the morning curves in Figs. 1 and 2 by some subjects without any glucose in the urine some criticism may perhaps be directed at our analytical methods. The modified Shaffer-Hartman method of sugar analysis we employed sometimes gives slightly higher values for the non-sugar fraction of reducing material present in blood than some of the other reagents. All determinations on blood-sugar reported in this paper, however, were made with one preparation of the reagent whose value to glucose remained constant.

A further series of 15 laboratory workers has yielded us results similar to those of Table II for fasting urine and after glucose ingestion. Some of these are reported in Table I. The occasional occurrence of a few mg. of fermentable sugar in the fasting urine in the morning may perhaps represent a carry-over from the meal of the previous evening, through incomplete removal of the night urine from the bladder, rather than any physiological variation, although we have met with one subject who excreted fermentable sugar in the fasting morning urine after a late meal (midnight), when the urine flow and intake of

water seemed sufficient to ensure thorough emptying of the bladder. This same subject showed minimal fermentable sugar excretion in the 7–9 a.m. specimen if the previous evening's meal was taken 6–7 p.m. In precise work the time relation of the previous evening meal and the fasting specimen cannot be disregarded.

In the remainder of the work reported in this paper we have taken a difference of 0·05 cc. (or over) of 0·005 *N* thiosulphate, between the total "sugar" and the non-fermentable "sugar" to mean the presence of fermentable sugar. Differences less than 0·05 cc. are reported as zero. By this procedure, perhaps, we have failed to observe the excretion of some small quantities of fermentable sugar; on the other hand, we have made certain that those amounts reported as present have a real significance.

Post-prandial.

The urines represented in Table III were collected 2–4 p.m. from a similar group of students. The urines were approximately 2 hours post-prandial. Half the results were obtained in November 1929 and half in October 1930.

Table III. *Fermentable sugar in post-prandial afternoon urine.*

Fermentable sugar mg./100 cc.	Fermentable sugar mg./100 cc.	Fermentable sugar mg./100 cc.	Fermentable sugar mg./100 cc.
7	7	5	4
0	0	19	14
0	5	12	0
0	36	17	0
0	0	16	20
6	0	18	8
0	0	11	6
7	?	0	0
0	6	0	10
21	0	0	0
16	0	0	0
12	13	0	0
0	0	4	8
33	8	10	12

The results, however, are the same. About 50 % of each group show unmistakably fermentable urinary sugar. Our results thus agree in general with those of Van Slyke and Hawkins, Hassan, Peterson and West, and Malmros, that it is not an uncommon finding. The significance of the remaining 50 % who show no fermentable sugar must not be overlooked. The negative results of Folin and Berglund, Eagle, and Greenwald, Gross and Samet could obtain equal support. Post-prandial urines from 8 normal women showed 4 containing fermentable sugar.

Several questions at once arise, the most important of which is the nature of the fermentable sugar. Glucose is not the only reducing sugar removable by yeast. Fructose and mannose are also fermentable and have been shown to be partially removable by yeast under the conditions of Raymond and Blanco [1928]. Fructose in aqueous solution can be distinguished from glucose and mannose by several reactions. The most notable of these is the Seliwanoff

reaction. The diphenylamine reaction has also been used in a quantitative manner by Van Creveld [1927] and Corley [1929]. Neither of these reactions yielded satisfactory results when applied to the urines containing fermentable sugar. We consequently fell back on indirect methods of approach.

Afternoon glycosuria.

In the endeavour to find conditions under which glucose might occur in normal urine the following experiment was performed on 5 laboratory workers. Fasting urine was collected 7–9 a.m. At 9 a.m. 50 g. glucose in water or weak tea were taken followed by ordinary breakfast. Two-hour specimens were collected throughout the day until 7 p.m. Lunch was taken at 1 p.m., and at 5 p.m. 50 g. glucose were again taken. Dinner was taken at 7 p.m. with a night specimen of urine from 7 p.m. till 7 a.m. next morning. In giving glucose with an ordinary breakfast we were influenced by the claims of Benedict and his co-workers [Benedict, Osterberg and Neuwirth, 1918; Benedict and Osterberg, 1923] that glucose taken with meals behaves quite differently from glucose taken in the usual fasting condition. In view of the clear difference just exhibited between fasting and post-prandial urines, it seemed worth while to re-study the question. Greenwald, Gross and Samet [1924] had found also, both in a human subject and in a dog, that glucose given with a high protein diet produced a glycosuria. Our reason for giving 50 g. glucose in the afternoon was the accidental observation, on one of us, that glucose taken at that time produced an unmistakable amount of fermentable urinary sugar, when none was present under conditions of the usual tolerance test. Three of the subjects (V. J. H.; D. L. S.; A. S. C.) had not known of any glycosuria before the test. L. J. H. was known to show sugar in the urine and had a familial history of diabetes, though no symptoms were present. C. E. D. had sometimes shown glycosuria after meals and sometimes was entirely free; there were, however, no symptoms and no history, personal or familial, of diabetes. All had normal fasting blood-sugars.

Table IV. *Afternoon glycosuria.*

	Fermentable sugar every 2 hours in subjects				
Time	V. J. H. mg.	D. L. S. mg.	A. S. C. mg.	L. J. H. mg.	C. E. D. mg.
7–9 a.m.	0	0	0	0	0
*9–11 „	0	0	0	7	22
11–1 p.m.	0	0	11	7	63
†1–3 „	0	0	7	39	23
3–5 „	0	0	9	7	8
‡5–7 „	132	0	0	174	277
§7–7 a.m.	0	0	0	—	0

* Breakfast + 50 g. glucose. † Lunch *ad lib.* ‡ 50 g. glucose. § Dinner *ad lib.*

The results are shown in Table IV. The two subjects known to show glycosuria excrete recognisable fermentable sugar after the breakfast. One has a few mg. of fermentable sugar during the middle part of the day only. The

remaining two excrete none. The contention of Benedict is evidently not universally applicable. 50 g. of glucose taken with breakfast will not ensure a glycosuria. Nevertheless in view of our own experience with glucose in the afternoon it is possible that his contention may be true of a larger number of people than our few examples would indicate.

Of great interest is the fate of the glucose taken at 5 p.m. In two subjects it evidently had no effect but in the other three it produced a large excretion. Even in subjects L. J. H. and C. E. D. who showed fermentable sugar after breakfast, the excretion was much greater after the afternoon dose. Most interest, however, centres about V. J. H. because (except for the result of a previous similar experiment) it was the first time fermentable sugar had been found in his urine. The zero excretions of fermentable sugar throughout the rest of the day show this subject to be neither a renal glycosuriac, nor a cyclic hyperglycaemic. Previous tests had also shown a normal glucose tolerance curve.

We have termed this phenomenon of glycosuria after a dose of glucose in the afternoon, when it is absent after a fasting dose of glucose, or increased glycosuria in the afternoon when it is present in the morning test "afternoon glycosuria."

For a long time we were uncertain of the real nature of the phenomenon, being unable to control its appearance or disappearance. Thus under ordinary conditions of diet D. L. S. never showed a trace of fermentable urinary sugar, morning or afternoon. V. J. H. showed the phenomenon twice, and then it disappeared on ordinary diets. We consequently carried out a large number of experiments on these subjects in an endeavour to trace some particular influence of the noon meal. These, though they proved failures from our immediate standpoint, led to another possible source of fermentable urinary sugar. Finally by using a new series of subjects, we were able to convince ourselves that afternoon glycosuria is a common occurrence and may represent the concomitant of a regular rhythm in the glucose tolerance.

Table V. *Showing excretion of fermentable sugar 2–4 p.m. and glycosuria 4–6 p.m. after 50 g. glucose at 4 p.m.*

No.	2–4 p.m. mg.	4–6 p.m. mg.	No.	2–4 p.m. mg.	4–6 p.m. mg.
1	12	0	10	2	90
2	19	23	11	0	0
3	12	9	12	12	6
4	0	6	13	6	76
5	0	0	14	0	7
6	26	56	15	0	4
7	0	0	16	6	0
8	0	5	17	6	86
9	4	10	18	6	25

In Table V are shown the fermentable sugar excretions of the 18 subjects of Table II, during the hours 2–4 p.m. and 4–6 p.m. Lunch (*ad lib.*) was to be taken at noon but we cannot be certain that this condition was kept absolutely.

At 4 p.m. they arrived at the laboratory and a glucose tolerance test with 50 g. glucose was carried out, similar to the usual morning one. In view of the claims of Lennox [1927, 1, 2] that the second glucose tolerance test always gives lower blood-sugar levels than the first, half our subjects received the morning test first, and half received the afternoon test first. A fortnight was allowed to elapse between the 2 tests on the same subject. The 2–4 p.m. specimens often showed significant amounts of fermentable sugar. In view of the uncertainty of the time of the midday meal, this may represent post-prandial sugar similar to the subjects of Table V. The significant results are 4–6 p.m. when 13 out of 18 show fermentable sugar. This under the circumstances can only be glucose. This is in sharp contrast with the results in Table II when only 1 out of 18 showed unmistakably a little glucose.

Blood-sugar curves at 4 p.m.

In Figs. 1 and 2 are shown the results of the blood-sugar determinations of the 18 subjects whose urinary fermentable sugars are reported in Tables II and V. There is one outstanding difference between the two sets of curves. In the afternoon series the peak of the curve is always higher than in the corresponding morning curve. In a few of the subjects the difference is so small that it could be counted as an experimental error, except that it is always in the same direction. In others, however, the difference is so marked that the results cannot be doubted. In 14 out of the 18 the difference is 20 mg./100 cc. or over. It seems evident to us that the higher peak of the afternoon curves represents a change in the tolerance of the individual towards sugar, either as a result of the day's activities, or as a sequence to the taking of food. It represents a change towards the condition designated as "cyclic hyperglycaemia" by the Scandinavian clinical workers or the "lag type curve" by the English. Some of the curves fulfil the criteria of "lag type" as defined by MacLean. There thus appears to be a greater chance of exceeding the renal threshold for sugar in the afternoon than under the usual fasting morning conditions of a glucose tolerance test. If the average figures for the peaks of our morning and afternoon curves are compared they are 159 and 191 mg./100 cc. glucose respectively. The average difference is not large, but at the peak of the curve it may be quite sufficient to be the cause of a glycosuria in the afternoon when none is present in the morning. It is to be noted that post-alimentary hypoglycaemia occurs in half the afternoon curves.

Are the results with glucose applicable to the occurrence of fermentable sugar in post-prandial urines? We believe they may be. Increases in blood-sugar are not obtained with glucose only. Bread [Jacobsen, 1913], potatoes [MacLean and De Wesselow, 1921; Rosenthal and Ziegler, 1929], rice [Kageura, 1922; Sakaguchi, Matsuyama and Watanabe, 1922], cooked starch [Foster, 1923], mixed meals [Kjer, 1924], have been used to study carbohydrate tolerance by means of blood-sugar findings. In general they give the same type of blood-sugar curve as glucose. The blood-sugar curve may remain below

the renal threshold for sugar, or may exceed it. Jacobsen [1913], using the Bang method of sugar analysis on finger blood, gave 157 g. of white bread to normal individuals and showed a rise in blood-sugar, sometimes to such an extent above the threshold that clinical glycosuria was evident. The test was given, sometimes fasting and sometimes 1½ hours after a light breakfast. There is thus no reason to exclude glucose as a possible post-prandial urinary fermentable sugar. At any rate it is certain from our experiments that the absence of glucose from the urine after its oral administration under fasting conditions in the morning is no argument for its non-appearance at other times during the day.

Our findings are against the conclusion drawn by many workers that the glucose tolerance curve is a constant for the individual [Hansen, 1923; Neilsen, 1928]. Such a conclusion we believe to be approximately correct for fasting conditions. Our morning tolerance curves show little inclination to rise above the usual threshold value. Under the shifting conditions of daily work and food, however, they vary. Moreover, a great deal of the work on which the idea of the constancy of the glucose tolerance test is based is itself open to criticism owing to the use of venous blood (now well known to give much lower peaks at the height of glucose ingestion [Friedenson, *et al.* 1928]), the collection of too infrequent blood samples to catch the peak blood-sugar value (even our 15 min. samples are barely sufficient), and to the use of relatively inexact clinical methods of detecting the glycosuria instead of methods of delicacy equal to those used in the blood analyses. This last reason has probably been a most potent factor in the lack of recognition of this variation in the glucose tolerance curve. All our urines were negative to Benedict's qualitative sugar reagent. With 30-minute blood samples probably only in 5 out of our 18 cases would there be sufficient difference in the afternoon curves to justify a differentiation from the morning curves. This general agreement, coupled with the negative Benedict test in the urine, would be sufficient to label all curves as normal in type, and the majority as approximately constant for the individual.

It might be objected that our results are brought about by the nature of the preceding meal or diet. Our subjects, however, were on their usual diet, and no restriction was placed on the nature of their noon meal. We obtained from each subject a list of the articles of food making up his lunch on the day of the afternoon test. They were very varied. The utmost that can be said is that the lunch was usually a light one. We have, however, precise experiments on the influence of the noon meal on the afternoon glucose tolerance test.

The nature of the noon meal and its effect on 4 p.m. glucose tolerance curves.

There has been a considerable amount of investigation on the effect of the previous diet on the ordinary glucose tolerance test as shown in blood-sugar curves. The most pronounced effect is that of fat contrasted with carbohydrate. High fat—low carbohydrate diets have been reported by Southwood [1923], Greenwald, Gross and Samet [1924], Sweeney [1927], Stenstrom [1927], Odin

[1927], Kohn, Fries and Felshin [1927], Malmros [1928] and Tolstoi [1929] to decrease the tolerance. Starvation [Bang, 1913; Traugott, 1922; Sevringhous, 1925; Pemberton and Foster, 1920; Du Vigneaud and Karr, 1925; Titiso, 1926; Harding and Van Nostrand, 1929; Hines, Boyd and Leise, 1929] where the body lives mainly at the expense of the fat depôts shows similar blood-sugar curves after glucose ingestion. High carbohydrate diets, conversely, increase the tolerance. The effect of protein is not so clear, Kageura [1922] had reported it to decrease the tolerance but Greenwald, Gross and Samet [1924], on an analysis of his diet, concluded that the effect was due to fat. Heinbecker [1928] found the glucose tolerance curves of the Greenland Eskimo living on a diet high in protein and fat to be normal. Sweeney [1927] found blood-sugar curves after 2 days' protein feeding to medical students intermediate in character between those obtained after a similar period of fat or carbohydrate feeding. On general grounds it might thus be expected that the character of the noon meal would influence the glucose tolerance test at 4 p.m. The only doubts that might exist would be the short time available for the effect of the meal, and whether one meal, however distinctive in character, would be sufficient to offset the influence of a previous ordinary diet. The shortest time in which a characteristic diet has been shown to affect a glucose tolerance test is recorded by Malmros who observed the change produced by a high fat diet for 1 day. We adopted 3 characteristic noon meals[1].

(1) *High fat meal:* 130 g. of 50 % cream made up partly as a cream soup, partly as whipped cream flavoured with saccharine, and partly taken in coffee.

(2) *High carbohydrate meal:* 150 g. carbohydrate in the form of potatoes, sweet potatoes, celery, lettuce, beets, carrots, tomatoes, and bread.

(3) *High protein meal:* 100 g. of protein in the form of roast veal and egg-white.

The subjects were laboratory workers; the noon meal was taken precisely at 12 noon; 2-hour urine specimens were collected commencing at 10 a.m. and the glucose tolerance test (50 g.) was commenced precisely at 4 p.m. An ordinary morning glucose tolerance test was performed on the subjects. A fortnight elapsed between each test. The fermentable urinary sugars are reported in Table VI, and the cutaneous blood-sugar curves on 6 of the subjects in Fig. 3. Venous blood-sugar curves on the remaining 2 subjects showed similar results but are not reported.

The urines show an almost remarkable paucity of positive results exclusive of the 4–6 p.m. period. None of the subjects shows fermentable sugar between meals. None is to be reckoned as a renal glycosuriac. It is not to be expected that a glycosuria should follow the high fat meal. No worker as far as we are aware has observed hyperglycaemia after fat. After protein or amino-acid feeding, however, rises in the blood-sugar have been noted. Cammidge, Forsythe

[1] The authors wish to express their thanks to Miss M. J. Porter and Miss L. Bryant of the Diet Kitchen of the Toronto General Hospital for their help, their unfailing courtesy, and the ingenuity with which they prepared the meals.

and Howard [1921] and Petren [1923] have noted hyperglycaemia after protein feeding. Pollak [1922] and Schatti [1923] found increased blood-sugar after amino-acids. Folin and Berglund, using venous blood for analysis and Lundsgaard [1930, 1, 2], on capillary blood, failed to find any increase in sugar either in man or in dogs after amino-acid or protein feeding. Rapport [1930] has recently reviewed this question from the general standpoint of the interconversion of foodstuffs. Our results, however, show no effect upon the urine in the form of fermentable sugar. It is after the high carbohydrate meal, however, that to us, the paucity of results possesses a possible significance. Only

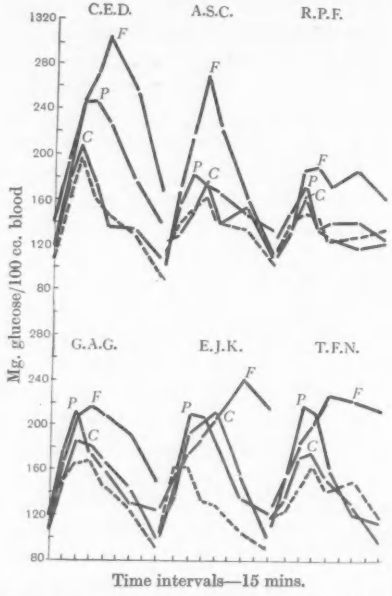

Fig. 3. Showing morning and afternoon tolerance curves after 50 g. glucose, with a variation in the nature of the noon meal.

Morning curves ------- Afternoon curves ———

C = Noon meal of carbohydrate. *P* = Noon meal of protein. *F* = Noon meal of fat.

2 out of 8 show glycosuria in the 12–2 p.m. specimen. In both of these the amount is considerable. In Table III the series of post-prandial urines shows 50 % of fermentable sugars. The amounts however are not large. In Table IV after mixed meals the subjects who show fermentable sugar show small or moderate amounts at hours other than immediately after meals. In Table V also a considerable percentage of subjects show small amounts of fermentable sugar. Our high carbohydrate noon meal contains 150 g. of carbohydrate almost exclusively in the form of starch. There is no cane sugar in the meal except a very small amount left after boiling the beets, carrots and sweet

Table VI. *Fermentable sugar in 2-hour urine specimen.*

Time	C. E. D. Noon meal of C. mg.	C. E. D. P. mg.	C. E. D. F. mg.	A. S. C. Noon meal of C. mg.	A. S. C. P. mg.	A. S. C. F. mg.	R. P. F. Noon meal of C. mg.	R. P. F. P. mg.	R. P. F. F. mg.	G. A. G. Noon meal of C. mg.	G. A. G. P. mg.	G. A. G. F. mg.
10 a.m.–12 noon	0	0	0	0	0	0	0	0	0	0	0	0
*12 noon–2 p.m.	141	0	0	0	0	0	0	0	0	0	0	0
2 p.m.–4 p.m.	0	0	0	0	0	0	0	0	0	0	0	0
†4 p.m.–6 p.m.	39	223	1696	12	16	275	0	5	0	8	34	317
Morning glucose tolerance test showed	6 mg.			0 mg.			0 mg.			10 mg.		

Time	E. J. K. Noon meal of C. mg.	E. J. K. P. mg.	E. J. K. F. mg.	T. F. N. Noon meal of C. mg.	T. F. N. P. mg.	T. F. N. F. mg.	V. J. H. Noon meal of C. mg.	V. J. H. P. mg.	V. J. H. F. mg.	D. L. S. Noon meal of C. mg.	D. L. S. P. mg.	D. L. S. F. mg.
10 a.m.–12 noon	0	0	0	0	0	0	0	0	0	0	0	0
*12 noon–2 p.m.	168	0	0	0	0	0	0	0	0	0	0	0
2 p.m.–4 p.m.	0	0	0	0	0	0	0	0	0	0	0	0
†4 p.m.–6 p.m.	80	67	479	0	42	367	0	131	738	0	10	0
Morning glucose tolerance test showed	0 mg.			0 mg.			0 mg.			0 mg.		

* Noon meal. † 50 g. glucose. C. = High carbohydrate. P. = High protein. F. = High fat.

potatoes. The fermentable sugar occurring in C. E. D. and E. J. K. after the high carbohydrate lunch must then almost certainly be glucose.

E. J. K. however shows a morning blood-sugar curve allowing the escape of no glucose, and illustrates our contention that the behaviour of the morning glucose tolerance curve is not an exact criterion of the action of carbohydrate at a meal later in the day. In contrast, G. A. G. shows 10 mg. glucose in the morning test and none after the carbohydrate meal. In general, however, the contrast we have drawn between the results shown in Table VI and other post-prandial results gives rise to the suspicion that fermentable sugar other than glucose can occur after meals.

The results of Table VI show afternoon glycosuria in 18 out of 24 experiments. Generally, it appears to be greatest after the fat and least after the carbohydrate noon meal. Two subjects (R. P. F., D. L. S.) show it only after protein, and it seems desirable to emphasise the fact that all the subjects have shown afternoon glycosuria following the noon meal of protein. The blood-sugar curves of Fig. 3 show in all 6 subjects a higher peak in the afternoon than the morning. Of the afternoon curves, those following the carbohydrate noon meal are the lowest, though in E. J. K. and A. S. C. the difference between the peak after carbohydrate and that after protein is very small. The results on the day of the high fat meal are of extreme interest. Three of the tolerance curves are almost diabetic in type. Only in 1 does the sugar value regain its initial value in 2 hours. There is thus not only a high peak but a prolongation in the tolerance curve. The 6 subjects who showed glycosuria gave a positive Benedict sugar test. These same 6 subjects showed marked ketonuria by Rothera's and Gerhardt's tests. It may perhaps be of significance that the 2 subjects (R. P. F. and D. L. S.) who showed no ketonuria also showed no glycosuria.

Malmros [1928] concludes that acidosis is not fundamentally related to the occurrence of glycosuria in non-diabetics but that it may be of importance as a supplementary factor. Our experiments are too few to throw much further light on this problem, but we should like to reserve this aspect of experimental ketonuria for future investigation.

The effect of protein appears intermediate between carbohydrate and fat. An interesting side-issue is the blood-sugar value at 4 p.m. after protein compared with its corresponding value after carbohydrate and fat. The figures are given in Table VII. With one exception, where the subject was continuously nauseated after the meal, the sugar values, both in cutaneous and venous blood were higher after protein than after carbohydrate or fat. In view of the results produced by individual amino-acids on blood-sugar levels in other animals this phenomenon may represent a stimulation of metabolism, rather than a new production of glucose. Lundsgaard [1930, 2] regards the hyperglycaemia from amino-acid feeding as a toxic effect. Also in view of the fact that our method of analysis determines the non-sugar reducing substances as well as true sugar, some uncertainty must attach itself to small differences.

Table VII. *Cutaneous blood-sugar at 4 p.m. after noon meal of protein, carbohydrate or fat.*

Mg. per 100 cc. blood.

Meal	C. E. D.	A. S. C.	R. P. F.	G. A. G.	E. J. K.	T. F. N.	V. J. H.*	D. L. S.*
Protein	140	111†	127	121	127	127	119	114
Carbohydrate	117	122	116	107	111	117	95	110
Fat	120	101	111	109	101	109	110	112

* Venous blood. † Nauseated.

There thus seems to be a good chance of a subject exhibiting glycosuria in response to 50 g. of glucose at 4 p.m. if the noon meal is of mixed character, and, under ordinary variations, the phenomenon will appear independently of the nature of the diet. Variations in individuals, however, are evidently to be expected. The reason for the disappearance of the phenomenon in V. J. H. noted above we now believe to have been an alteration in the glucose tolerance due to repeated experimental administration of glucose which is reported by several observers to improve the tolerance. Eisner [1926] fed glucose daily to rabbits and noted the production of a low type glucose tolerance curve. John [1922] gave 100 g. of glucose to two normal individuals on 5 successive days. The blood-sugar peak on the 1st day was 260 mg./100 cc. glucose; on the 5th day it was 90 mg./100 cc. Sansum, Blatherwick and Bowden [1926], Gibson [1929] and Rabinowitch [1930] have all recently reported the stimulating effect of glucose or carbohydrate feeding on the carbohydrate tolerance of diabetics. It seems very probable that continued administration of glucose may affect also the afternoon glucose tolerance curve. It was a recognition of this possibility that led the authors to select 18 medical students as an entirely new series of subjects for the demonstration of afternoon glycosuria as a common phenomenon.

We can find only a few odd references in the literature bearing on afternoon glycosuria and the corresponding blood-sugar curves. Staub [1921] states that if 20 g. glucose are given 5 hours after a meal, the blood-sugar curve is higher than after a similar dose taken 5 hours later again. Another possibility of rhythm in glucose tolerance may perhaps be found in the work of Kohn, Fries and Felshin [1927], who reported that 2 or 3 days after the change from a high fat diet to a high carbohydrate diet, the glucose tolerance test showed abnormally low blood-sugar curves, and only later gave curves of more normal altitude. This was found in children but has not been noticed by other observers working on adults. The phenomenon may perhaps also have occurred in some of the experiments of Folin and Berglund [1922, 2] for they remark that they "are under the impression that excessive hyperglycaemias are more easily obtained in the evenings than during the early forenoon hours."

Our observations on afternoon glycosuria and its cause apparently bring us into conflict with a widely held view that the carbohydrate tolerance, both of normals and diabetics is lowest in the morning. Naunyn is quoted by Folin and Berglund [1922, 2] as giving this statement as a general expression of clinical opinion on the tolerance of the diabetic in 1906. Peters and Van Slyke [1931] use the morning rise in blood-sugar in the diabetic as illustrating the necessity for the insulin dosage to be highest in the morning. The lowered tolerance of the severe diabetic in the morning may represent one of the differences between that disease and the normal. Such evidence as we have seen, as that of Page [1923], Jonas, Miller and Teller [1925] and Watson [1929], however, by no means inclines us to the view that the normal shows the same lowered morning tolerance. Nor do we wish to be put on record as stating that 4 p.m. blood-sugar tolerance curves are always to be found with a higher peak than those at 9 a.m. in normal individuals. We merely state that such is a common occurrence and leave any discrepancy between our results and those of other observers on normal carbohydrate tolerances to future investigation.

Objection may perhaps be raised to our description of some of the 4 p.m. blood-sugar curves as "lag type." The blood-sugar curves themselves fulfil the criteria of the "lag type" curve. The glycosuria which accompanies our curves, however, is minute compared with "clinical" glycosuria. This difference, however, is only one of degree and we believe may be due to the clinical observations being made on individuals possessing a renal threshold for sugar lower than the usual normal average. Thus a subject possessing a renal sugar threshold of 100–120 mg./100 cc. would show no sugar in the fasting urine with the ordinary clinical tests. After 50 g. glucose, with a usual rise of blood-sugar to 170–180 mg./100 cc. he might show a positive Benedict test. He would then be classed as a renal glycosuriac of the intermediate type described by Graham [1923]. Should his blood-sugar peak rise to 200–220 mg./100 cc. or over it is almost certain that urinary sugar would be found. He would then be classed as a "lag type." Although this may be the explanation of the clinical glycosuria accompanying the "lag type" curve, it does not explain why such

individuals show a "lag type" curve in the morning while our normals only show the high peak in the afternoon.

Urinary fermentable sugar after fruits, honey, orange juice and invert sugar.

In the preceding sections of this paper we have given cogent reasons why glucose may at times be a constituent of normal urine. At the same time doubts have been expressed on the single nature of the urinary fermentable sugar after meals under all conditions. These suspicions were engendered by observations on the authors in their preliminary search for conditions under which afternoon glycosuria might be demonstrable. They took a series of noon meals of varied character. The high fat, protein and carbohydrate, with their negative results, have already been discussed in connection with Table VI. In addition, the effect of variations in the proportions of protein, fat and carbohydrate were studied, using the same food materials. Entirely negative results were obtained. When, however, in high carbohydrate meals a large proportion of the carbohydrate arose from fruits, small amounts of fermentable sugar made their appearance in the urine. The details of some of the meals and the results of some of the experiments are shown in Table VIII. In all, 19 experiments of this nature were performed and fermentable urinary sugar was

Table VIII. *Excretion of fermentable sugar after meals containing large amounts of fruit.*

Expt.	Character of noon meal		Subject	Fermentable sugar: 12 noon–2 p.m.	Fermentable sugar: 2–4 p.m.
1	High protein and fat	(*a*)	V. J. H.	0	0
			D. L. S.	0	0
2	(*a*) + 60 g. cane sugar		V. J. H.	0	0
			D. L. S.	0	0
3	Mixed	(*b*)	V. J. H.	0	0
			D. L. S.	0	0
4	Mixed	(*c*)	V. J. H.	0	0
	Mixed	(*d*)	D. L. S.	0	0
5	Carbohydrate (fruit salad)	(*e*)	V. J. H.	24	5
			D. L. S.	12	8
6	Carbohydrate (fruits)	(*f*)	V. J. H.	15	0
			D. L. S.	25	0
18	Carbohydrate (fruits)	(*g*)	V. J. H.	18	0
			D. L. S.	8	11
19	Carbohydrate (fruits)	(*h*)	V. J. H.	30	0
			D. L. S.	?	0

(*a*) Oysters, sirloin steak, rolls, butter, coffee, cream, sugar.

(*b*) Lamb, potatoes, carrots, bread, butter, apple pie, tea, cream, sugar.

(*c*) Beef, potatoes, cheese, bread, butter, coffee, apple pie, jelly.

(*d*) Soup, veal, potatoes, corn, bread, butter, cream, coffee.

(*e*) Salad composed of potato, celery, apple, cherries, nuts, mayonnaise; bread, butter, apple pie, cheese, coffee, sugar.

(*f*) Grapefruit, prunes, apricot, peaches, bread, butter, apple pie, coffee, cream, sugar (50 g.).

(*g*) 150 g. potato, 80 g. carrot, 70 g. sweet potato, 120 g. orange (edible portion), 100 g. banana (edible portion), 200 g. orange juice, cream, coffee.

(*h*) Largely orange and banana, small amount potato and carrot, 200 g. orange juice.

clearly demonstrated only in 4, *viz.* when the meal contained a large amount of fruit. Since neither author is in the habit of showing demonstrable urinary fermentable sugar after ordinary meals, the positive results after fruit, in both authors, and occurring under the same conditions, become thus more remarkable and point to the occurrence of fermentable sugar in urine other than glucose.

We extended our observations to two other subjects; one of whom, judging by the results in Table VI, possesses as efficient a mechanism as D. L. S. for the retention of glucose. To these subjects we gave 500 cc. of orange juice at 9 a.m. under the conditions of a morning glucose tolerance test. The juice was made from 12 Florida oranges, extracted the previous afternoon, and kept overnight in a refrigerator. It represents 50 g. edible carbohydrate. The results are shown in Table IX. Both showed fermentable urinary sugar. A repetition of the experiment gave the same result. Honey, and 50 g. of invert sugar prepared in the laboratory by the action of 0·5 *N* HCl on cane sugar, also gave fermentable urinary sugar. Addition to the orange juice of (*a*) glucose, (*b*) cane sugar, (*c*) invert sugar did not increase the urinary fermentable sugar. 50 g. cane sugar gave negative results. In view of this result we observed the action of glucose which had been allowed to stand overnight with 0·5 *N* HCl, in case the action of the acid might produce small amounts of fermentable material which escaping into the urine would be reckoned as glucose, though it is more usual to suppose the stability of sugars under such conditions. The result of the experiment was negative.

Table IX. *Excretion of fermentable sugar after orange juice, honey and invert sugar.*

Expt.	Ingestion at 9 a.m. of	Subject	Fermentable sugar 7 a.m.–9 a.m.	9 a.m.–11 a.m.	11 a.m.–1 p.m.
			mg.	mg.	mg.
20	500 cc. orange juice	R. P. F.	0	7	0
		C. E. D.	0	7	0
21	500 cc. orange juice + 40 g. cane sugar	R. P. F.	0	12	0
		C. E. D.	0	12	0
22	90 g. cane sugar in 500 cc. water	R. P. F.	0	0	0
		C. E. D.	0	0	0
23	500 cc. orange juice	R. P. F.	—	5	0
		C. E. D.	0	15	0
24	500 cc. orange juice + 40 g. glucose	R. P. F.	0	13	0
		C. E. D.	0	7	0
25	500 cc. orange juice + 40 g. partly hydrolysed cane sugar	R. P. F.	0	18	6
		C. E. D.	0	28	0
26	500 cc. orange juice + 40 g. invert sugar	R. P. F.	0	0	0
		C. E. D.	0	15	0
27	90 g. invert sugar in 500 cc. water (50 cc. *N*/2 HCl)	R. P. F.	0	8	0
		C. E. D.	0	10	0
28	120 g. honey in 500 cc. water	R. P. F.	0	5	0
		C. E. D.	0	15	5
29	50 g. glucose + 50 cc. *N*/2 HCl in 500 cc. water	R. P. F.	0	0	0
		C. E. D.	0	0	0

Such a series of experiments could point only to the presence of free fructose, some decomposition product by acid, or a new substance removable by yeast and formed as a metabolic product from fructose. We feel our experiments may offer a partial explanation of some of the results in the literature on post-prandial sugar. The use of jams, preserves, and marmalade, where acid fruits are boiled with cane sugar, might offer another source of urinary fermentable sugar.

Urinary fermentable sugar after fructose.

On six subjects we examined the action of 50 or 25 g. of fructose both at 9 a.m. under the usual fasting conditions, and at 4 p.m. The fructose used was Schuchart's "puriss. cryst." ($[\alpha]_D^{20} - 92{\cdot}5°$). The urine findings are given in Table X and the capillary blood-sugars in Fig. 4.

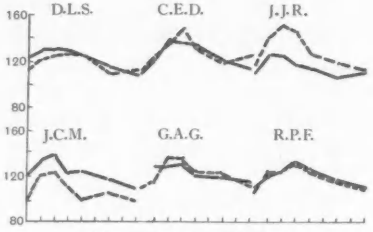

Fig. 4. Showing morning and afternoon tolerance curves after 50 or 25 g. of fructose.
Morning curves -------- Afternoon curves ————

All subjects showed fermentable urinary sugar both in the morning and afternoon. The amounts are small, and are of the same magnitude as those shown after fruits, *etc.* in the preceding section. Moreover in the morning experiments there is seen an excretion lasting 2–4 hours after the administration of the sugar in three of the six subjects. A similar continued excretion is noticed in some of the experiments after fruit, *etc.* and in some of the post-prandial sugars. We have referred to those in Table IV. Some of those in Table V may also represent a similar continued excretion. Recently Corley [1929] has shown the presence of small amounts of urinary fructose after fructose feeding in the rabbit. The presence of fructose in the blood-stream after fructose feeding has been shown in man by Folin and Berglund [1922, 2] using the Seliwanoff test, and by Corley in the rabbit using the modified Van Creveld method. There thus seems no reason to refuse to label the urinary fermentable sugar in our experiments after the feeding of fructose, fruits, honey or invert sugar, as fructose, unless some unidentified metabolic product behaves in a similar way. That fructose differs from glucose in its metabolism is well known. It possesses its own rate of absorption [Cori and Cori, 1925]. Its high ability to form glycogen [Cori, 1926], its poor utilisation in the muscles [Mann and Bollman,

1930] and the accompanying formation of lactic acid after oral administration [Campbell and Maltby, 1928] all mark it off from glucose.

Table X. *Urinary fermentable sugar in six subjects after oral administration of fructose at 9 a.m. and at 4 p.m.*

Subject	Fructose ingested	Fermentable sugar in 2 hr. urine: Fructose at 9 a.m. 9–11	Fructose at 9 a.m. 11–1	Fructose at 4 p.m. 4–6
	g.	mg.	mg.	mg.
D. L. S.	50	7	4	8
C. E. D.	50	7	0	8
J. J. R.	25	21	0	13
J. C. M.	25	14	0	13
G. A. G.	50	19	10	13
R. P. F.	50	8	0	9

The differentiation from glucose is also to be noticed in the shape of the blood-sugar curves after ingestion. The total blood-sugar is only elevated a little in comparison with the rises noted after glucose. The morning curves shown in Fig. 4 confirm many observations in the literature. The afternoon curves are almost exactly similar to the morning curves. There is no evidence of any afternoon fructosuria either from the urinary findings, or from any increased peak or height in the blood-sugar curves. This again differentiates its metabolism from glucose. At present we do not wish to offer any explanation, either of the phenomenon of afternoon glycosuria or of our failure to observe a similar phenomenon after fructose.

General conclusions.

1. Within the limits of the analytical methods fermentable sugar is absent from normal fasting urine. The possible amounts of fermentable sugar must be under 5 mg./100 cc. and are more likely to be less than 2–3 mg./100 cc. We have excluded known renal glycosuriacs from our observations.

2. After glucose ingestion in the morning under fasting conditions no increased amount of fermentable sugar can be detected in the urine. A survey of a large number of individuals will, however, undoubtedly reveal some showing glycosuria, though too small to be detected by the usual clinical reagents. The percentage in which such individuals occur in a series will influence the experimenter to declare the presence or absence of glucose in normal urine after glucose ingestion. Three such examples occur in our experiments. In one there appears to be a slightly lowered renal threshold.

3. 50 % of medical students show small amounts of fermentable sugar in the post-prandial afternoon urine.

4. Two subjects who showed no fermentable urinary sugar, fasting, after glucose in the morning, or after ordinary mixed meals, showed fermentable sugar after large amounts of fruit.

5. Fermentable urinary sugar was also shown to occur in two other subjects after orange juice, honey and invert sugar, taken fasting.

6. 50 or 25 g. of pure fructose given as a fructose tolerance test in the morning or in the afternoon at 4 p.m. give rise to urinary fermentable sugar.

7. 50 g. glucose at 4 p.m. give rise to glycosuria in individuals showing none under the fasting conditions of the morning test. This phenomenon of afternoon glycosuria is of frequent occurrence. It is least likely to occur if the noon meal is high in carbohydrate. It has always occurred if the noon meal is high in protein. A high fat noon meal is also very likely to cause afternoon glycosuria.

8. The blood-sugar curves at 4 p.m. taken under ordinary conditions of diet all possess a higher peak than the corresponding fasting morning tolerance curves. The peak often rises above the usual renal threshold for sugar. This accounts for afternoon glycosuria. Some of the blood-sugar curves resemble those of the MacLean "lag type."

9. The nature of the noon meal affects the blood-sugar curve after 50 g. glucose at 4 p.m. A high carbohydrate meal gives rise to the lowest curve. A high fat meal gives rise to a high peak curve, sometimes remaining high at the end of 2 hours. Ketosis was observed in six out of eight experiments after our high fat noon meal. A high protein noon meal gives a high peak blood-sugar curve. All tolerance curves at 4 p.m. are higher than the morning tolerance curve in the same individual.

10. We have found no phenomenon corresponding to afternoon glycosuria in the urine, or in the shape of the blood-sugar curves, after the oral administration of fructose. This may serve as additional evidence differentiating the metabolism of glucose from that of fructose.

11. The non-occurrence of glycosuria after the usual morning glucose tolerance test cannot be taken as a criterion of its continued non-occurrence throughout the day. After a noon meal where the carbohydrate consisted almost entirely of starch a subject showed glycosuria, although a morning tolerance test with glucose was entirely negative. Another subject showed a few mg. of glucose in the urine after the morning test, but none after the carbohydrate meal.

12. The occurrence of fermentable sugar in normal urine is a variable phenomenon depending on a number of complex factors.

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CXCVII. A MODIFICATION OF THE METHOD OF STEWART AND WHITE FOR THE DETERMINATION OF BLOOD-FAT, WITH OBSERVATIONS ON SEVERAL SPECIES IN POSTABSORPTIVE CONDITIONS.

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The present report contains a study of the method of Stewart and White [1925] for the determination of blood-fat, and full details are given concerning a modification which, in our hands, has proved more practicable than their original procedure. Substances interfering with the determination have been examined and the error of the method has been determined. The data also include observations on the fluctuations occurring in a given animal and the variations in the level of blood-fat of a series of animals of several species in the postabsorptive state.

Procedure.

Preparation. 5 cc. samples of oxalated blood are centrifuged for half an hour and 1 cc. of the plasma thus obtained is added to 35 cc. of an alcohol-ether mixture (3 parts 95 % alcohol—1 part ordinary ether). By adding the plasma drop by drop while constantly rotating the flask, a finely divided precipitate is obtained.

Extraction. The flask is plunged in a bath of boiling water. The mixture is boiled for a few seconds, cooled to room temperature and filtered into a 50 cc. volumetric flask. If the fat content of the blood is high, the accuracy is increased by making the alcohol-ether extract up to 100 cc. instead of 50 cc.

Saponification. Aliquot portions (15 cc.) of the filtrate are measured into 50 cc. Erlenmeyer flasks and controls are made on the same volume of alcohol-ether mixture. The mixture is evaporated to about 1 cc., then 5 cc. of 0·1 *N* NaOH are added from a burette and the mixture is again evaporated to approximately 1 cc. In order to complete the saponification, 5 cc. of absolute alcohol are added, and evaporation is continued until 2 cc. of the solution remain. A hot plate is always used for evaporation.

[1] Some of the data of this paper are taken from an essay presented by Hilda Friedman in candidacy for the degree of Master of Science, Yale University, 1928.

Acidification. Liberation of fatty acids. 5 cc. of 0·1 *N* HCl are added. This should exactly neutralise the alkali previously combined and liberate the fatty acids. The mixture is then boiled down to half its volume in order to expel any absorbed CO_2.

Titration. After adding 10 cc. of absolute alcohol, the solution is heated and titrated while hot with 0·02 *N* NaOH, using phenolphthalein as indicator.

Calculation. If 1 cc. of plasma is dissolved in 50 cc. alcohol-ether mixture and 15 cc. of the extract are used, the final aliquot corresponds to $1/50 \times 15$ or 0·3 cc. of plasma. With a final titration value for 0·02 *N* NaOH of *X*, the amount of fat present in 100 cc. of plasma is equal to $X \times 100/0{\cdot}3 \times 5{\cdot}38$; 5·38 being the factor when calculating in terms of tripalmitin. In a determination of fat, the titration value of the control is subtracted from that of the unknown and the difference is multiplied by the factor 1793 in order to determine the fat present in the blood sample in mg./100 cc. The result so obtained is then corrected for any change in concentration.

Recovery of the higher fatty acids. Accurately weighed amounts of pure palmitic and oleic acids were dissolved in definite volumes of the alcohol-ether mixture and determinations were made on portions of these solutions. The procedure for determining these samples was similar to directions given above for the treatment of plasma. The recovery of these fatty acids is given in Table I.

Table I. *Recovery of known amounts of fatty acids.*

Fatty acid	Amount present mg./100 cc.	Amount recovered mg./100 cc.	% recovered	Deviation of recovery
Palmitic	340	337·9	99·4	−0·6
Palmitic	888	900·0	101·0	+0·1
Palmitic	622	611·0	98·2	−1·8
Oleic	340	315·8	92·5	−7·5
Oleic	240	236·0	98·3	−1·7
Mixture, oleic + palmitic	410	406·0	98·0	−2·0
Mixture, plasma extract + oleic + palmitic	376	400·0	106·0	+6·0
			Average	±2·52

Interfering substances. A number of observations were made to determine whether lactic or butyric acid, or their sodium salts, or acetone acted as interfering substances in the estimation of fat in the blood. 1 cc. of solution containing 1·2 mg. of lactic acid was dissolved in the alcohol-ether mixture and made up to 50 cc. 1 cc. of solution containing 0·96 mg. butyric acid was similarly treated. Determinations were made on 15 cc. aliquots of these solutions using the same procedure as in the treatment of plasma. Similar determinations were made using sodium lactate, and butyrate, and acetone. None of these substances could be titrated by this method.

Constancy of recovery. It seemed advisable to estimate the change that might occur in blood drawn at short intervals of 2 minutes, and then again at intervals of 2 hours. Results have shown that blood obtained from dogs anaesthetised with amytal and in the postabsorptive state at intervals of

2 minutes and determined separately remained practically unchanged, the average deviation of a single determination being ± 0·4 %. The values of fat in blood taken at 2-hourly intervals for 8 hours, in the case of three such dogs, have shown that there is an average deviation of ± 2·2 %. In the case of unanaesthetised dogs the average deviation of a single determination was ± 3·5 %. Four of these determinations were corrected for total solids and yielded an average deviation of ± 3·7 % after the correction was made (average percentage deviation = ± 3·6). Repeated observations of the same animals over a period of 6 months are presented in Table II.

Table II. *Repeated observations on the same animal.*

Dog	Number of observations	Average fat value mg./100 cc.	Average % deviation
I	7	663	±15·30
II	7	692	±15·00
III	4	720	±20·60
IV	4	656	±13·00
V	2	767	±13·25
VI	2	675	±32·00
VII	2	691	± 2·91

Values under postabsorptive conditions. The average fat values for normal resting animals under postabsorptive conditions have been calculated from a large number of determinations and are as follows: dog 673, cat 611, monkey 501, rabbit 498, man 481 mg./100 cc. respectively.

Discussion.

The changes from the technique of Stewart and White. The method described above differs in some respects from that originally presented by Stewart and White [1925]. In the first place, we have found it possible to get satisfactory results using 1 cc. of whole blood or 1 cc. of blood-plasma instead of 2 cc. as indicated by Stewart and White [1925]; thus less blood is required. Determinations have been made of the fat content of 1 cc. of whole blood and 1 cc. of plasma from the same blood sample. In some instances there has been a difference between the fat content of the whole blood and that of the plasma. Therefore, blood-plasma instead of whole blood was used whenever possible, since the red blood corpuscles may vary in the course of an experiment and thus yield an erroneous result.

According to Stewart and White [1925], the alcohol-ether extract of plasma is brought to the boil and made up to volume. In order to withdraw the supernatant fluid, the mixture is allowed to stand so that the precipitate may settle. There are several objections to this procedure. Frequently the supernatant fluid is not entirely clear. Again, the fluid withdrawal may not be uncontaminated with the precipitate. Finally, the amount of filtrate obtained is limited by this method of withdrawal. For these reasons, filtration was substituted for decantation as described above. The extraction of lipins with filtering was practically complete because plasma was used. To quote Bloor

[1928], "the statement appearing in the literature and attributed to the author that the alcohol-ether extraction is not complete by this procedure (filtering) applies only to whole blood or corpuscles and not to plasma." A hot plate is preferable to a steam-bath for boiling down the various mixtures after each step in the process.

In the final step, according to Stewart and White [1925], 2 cc. of the final solution (composed of fatty acids and 10 cc. absolute alcohol) is equivalent to 1/10 of the original sample and is transferred and titrated with 0·1 *N* NaOH from a Rehberg burette. In the present modification of the method the total final solution is used for the titration. This avoids any error of transference.

In our analyses a micro-burette, with a very fine tip, and a capacity of 1·5 cc. is used. This burette is graduated in 0·01 cc. divisions, so that it is possible to interpolate to 0·001 cc. Checks within 0·005 cc. are frequently obtained.

It is found that on titrating in the manner suggested by Stewart and White [1925], with CO_2-free alkali, the CO_2 in the air interferes to such an extent as to give uncertain end points. The titration was therefore conducted at the boiling-point as described above. Furthermore, the absolute alcohol itself is so acid as to require almost as much of the alkali for its neutralisation as is necessary for an equal volume of fatty acid solution with the small quantities measured by Stewart and White [1925]. Attempts to rid the alcohol of its acidity by distilling over lime and caustic alkali were unsuccessful. The error due to alcohol is eliminated by the use of control determinations on the reagents.

As larger amounts of the more dilute alkali are used, the error of titration may be lessened. Therefore, 0·1 *N* NaOH was replaced by 0·02 *N* NaOH.

It became necessary to make a correction for changes in concentration of successive samples of plasma from the same animal, for the fatty acids determined by this method are present in the plasma as esters and chiefly in suspension in the form of triglycerides, only a small portion existing in solution as phospholipins [Bloor, 1916, 1, 2; Boyd, 1931]. This correction was made by drying a 1 cc. portion of each sample of plasma to constant weight at 100°. Thus it was possible to estimate any change in the water content of the plasma.

Table I shows the percentage recovery of the original amount of palmitic acid to be 99·5 %, of oleic acid 95·9 %, of a mixture of palmitic and oleic acids 98 %, and of a mixture of plasma extract and palmitic and oleic acids 106 %. The average deviation is ± 2·52 %, and all results are within 5 % of the theoretical values.

Error of method. In our estimations triplicate portions were used to determine the fatty acid content of the blood-plasma sample, and the best two of the three titrations were used for calculation. In this manner, the error of the method, taking the average of 266 triplicate determinations, was found

to be ± 11 mg./100 cc. This value is equal to a deviation of ± 1·6 % from the average for resting dogs (673 mg./100 cc.). For the maximum and minimum values the error is ± 1·1 % and ± 2·8 % respectively.

The average values varied with the species. Those found for man by Wilson and Gildea[1] (481 mg./100 cc.), using the present method are not so different from those of Nicholls and Perlzweig [1927–28] who determined the fat content of human blood using a modification of the method of Stewart and White [1925] and found it to be 426 mg./100 cc. Stoddard and Drury [1929], using their own method, also worked on human beings and reported an average fatty acid content of 294 mg./100 cc. in a series of 10 blood samples. Bloor *et al.* [1922] employed his nephelometric method and found an average value of 590 mg./100 cc. for dogs, while with his newer oxidation method [1928] he obtained slightly higher values. All these investigators used methods based on that of Kumagawa and Suto [1908] who employed a solvent for fatty acids to extract them from the blood, the subsequent steps, however, differing according to the various modifications.

The constancy of recovery obtained by our method has been shown above.

Results obtained by other methods yield the same general implications as to the deviations in blood-fat. The average deviation of a series of animals observed by Terroine [1914–15] at weekly intervals was ± 4·5 %, but the widest differences were ± 30 %. Bloor [1914] found the greatest variation of any animal to be ± 12 %, while the average was ± 6 %. Patterson [1927] noted that the fasting fat content for given individuals over a considerable period of time exhibited but slight variations, the widest being ± 6·5 % with an average deviation of ± 3 %.

Observations of a series of animals under postabsorptive conditions. In a large number (266) of observations on various animals, the minimum fat value for a normal resting dog was found to be 428 mg./100 cc., while the maximum was 906 mg./100 cc. This indicates a variation of 111 % for different individuals. Terroine's [1914–15] results were not unlike ours, as he found the extreme values to be 314 mg./100 cc. and 669 mg./100 cc. respectively, the variation being, therefore, 113 %. According to Bloor [1914], the average deviation of any animal in his series was ± 7 %, the greatest being ± 14 %, while Nicholls and Perlzweig [1927–28] give the blood values as varying between 333 and 492 mg./100 cc. in human subjects.

Summary.

A modification of the method of Stewart and White [1925] for the determination of blood-fat is presented. The experimental error of this method is ± 11 mg./100 cc., which represents a percentage deviation of ± 1·6, since 673 mg./100 cc. is the average value for dogs in the postabsorptive state. The level of blood-fat varied by ± 3·6 % in the course of an observation period

[1] Personal communication from Doctors E. F. Gildea and W. R. Wilson, who obtained six and three results respectively on normal human subjects.

of 8 hours, and therefore a difference of 40 mg./100 cc. has been considered significant. It was found that the values for blood-fat in postabsorptive conditions not only varied widely (111 %) in a given species (dog), but that the average values differed in various species. From an examination of the interfering substances it has been revealed that the method does not estimate the fatty acids which exist in the blood as soaps, but only those in ester combination.

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CXCVIII. THE MICRO-DETERMINATION OF SODIUM IN BIOLOGICAL MATERIALS.

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In a recent paper McCance and Shipp [1931] described a colorimetric method for determining very small amounts of sodium (0·02–0·8 mg.). The present paper describes the application of this method to the various body-fluids and solid tissues. By employing suitable protein precipitants the necessity for incineration has been avoided, except in the case of solid organs. This has great advantages: (1) in dealing with very small amounts of material; (2) there is always a risk of losing sodium by volatilisation; (3) there is a great saving of time; (4) no special apparatus is required.

(1) *Whole blood and serum.* Barrenscheen and Messiner [1927] described the determination of sodium in serum by means of a uranyl zinc acetate precipitation without incineration, but in our hands their technique has not proved satisfactory.

Grigaut and Boutroux [1930] have published a method based on the removal of the proteins by alcoholic uranium acetate. The sodium is then determined gravimetrically or volumetrically. Their results tend to be too high owing, they state, to the space occupied by the precipitated proteins. They do not state whether volume changes due to the addition of alcohol may not also contribute to the high results.

We have used trichloroacetic acid to precipitate the proteins. At the acid reaction the proteins are not precipitated as their sodium salts. The procedure is as follows. To about 8 cc. of water in a 25 cc. flask add 0·5 cc. of serum (or 1 cc. of whole blood). Add then with shaking 1 cc. of 30 % trichloroacetic acid (or 3 cc. in the case of whole blood). Allow to stand for 5 minutes, make up to the mark, mix and filter through an ash-free paper. It is important not to have too large a volume of liquid in the flask before adding the trichloroacetic acid. 1 cc. of the filtrate is then taken for each estimation and the sodium determined exactly as described in the previous paper. The sodium precipitate should be washed into 25 cc. flasks for colorimetric comparison. In Table I will be found a series of comparisons between this method of removing the blood- or serum-proteins and an incineration method. For the latter 1 cc. serum or 2 cc. whole blood were subjected to incineration in a silica crucible just below dull red heat, the product extracted 5 times with boiling $N/2$ HCl and the whole made up to 50 cc.

Barrenscheen and Messiner [1927] directed in their paper that the serum-proteins be removed by an alcoholic zinc acetate reagent which removes the phosphates at the same time. Though this eliminates one stage of the method, we have not found it satisfactory, since, after removal of the proteins with alcoholic zinc acetate, the precipitation of the sodium is very slow and erratic; and, in one or two cases, we have found that no sodium at all was precipitated in 1 hour. If left for a long time the precipitation of the sodium by the uranyl zinc acetate is probably complete, but the precipitate sticks to the sides of the tube, and centrifuging and washing are not so easy as they are after incinerating or employing trichloroacetic acid. Recoveries of sodium added to serum and blood by the trichloroacetic acid method will be found in Table II.

Since lithium interferes with the estimation of sodium as sodium uranyl zinc acetate [Caley and Foulk, 1929] lithium oxalate must not be used as an anticoagulant. We have used ammonium oxalate for our whole blood determinations.

(2) *Cerebrospinal fluid.* The technique is exactly the same as for blood-serum. Although normal cerebrospinal fluid contains practically no protein, we employ trichloroacetic acid and filter if there is visible opalescence. We give in Table II some recoveries of varying amounts of sodium added to cerebrospinal fluids.

(3) *Urine.* In urine there is usually enough sodium available to enable macro-methods to be used and Poulsson's [1928] modification of Barrenscheen and Messiner's method [1927] is probably quite satisfactory. For investigations on small experimental animals or even insects a micro-method is desirable. We have accordingly tested our procedure on urines and give in Table II some of our recoveries of known amounts of sodium added to urine. An accurate 1 in 25 or 50 dilution of the urine is prepared. 1–2 cc. of this diluted urine are then taken for each determination which is carried through as previously described.

Table I. *Estimation of sodium.*

Material	By incineration mg. per 100 cc.	By direct precipitation mg. per 100 cc.
Serum	348	346
,,	391	394
,,	330	340
,,	344	341
Whole blood	167	177
,,	212	208
Human milk	36·4	38·4
,,	42·5	42·7
,,	45·7	47·0
Cow's milk	89·3	89·3
,,	89·9	92·5

(4) *Milk.* As in the case of blood the successful determination of sodium without incineration depends upon the use of a suitable precipitant for the milk proteins. Bromine was suggested as a protein precipitant by Rideal and Stewart [1897] and was more thoroughly investigated by Allen and Searle

Table II. *Recovery of added sodium.*

Material and amount to which addition was made		Na added mg.	Na recovered mg.	Na recovered %
Serum	0·5 cc.	1·00	1·04	104·0
,,	0·5 ,,	2·00	2·11	105·5
,,	0·5 ,,	1·00	1·02	102·0
,,	0·5 ,,	2·00	1·96	98·0
,,	0·5 ,,	3·00	2·85	95·0
,,	0·5 ,,	1·20	1·17	97·5
,,	0·5 ,,	2·00	2·08	104·0
,,	1·0 ,,	4·00	4·20	105·0
Whole blood	1·0 ,,	1·00	0·96	96·0
C.S.F.	0·5 ,,	1·00	1·03	103·0
,,	0·5 ,,	1·00	0·99	99·0
,,	0·5 ,,	2·00	1·92	96·0
Urine	1·0 ,,	2·00	1·90	95·0
,,	1·0 ,,	2·00	2·04	102·0
,,	1·0 ,,	2·00	2·04	102·0
,,	1·0 ,,	2·00	1·95	97·5
,,	1·0 ,,	0·67	0·64	95·0
Milk (cow)	5·0 ,,	4·00	3·97	100·5
,, (human)	2·0 ,,	0·8	0·805	100·5
,, ,,	2·0 ,,	1·5	1·57	104·5
,, ,,	2·0 ,,	2·0	1·98	99·0
Fish	20 g.	10·0	10·0	100·0
,,	20 ,,	10·0	9·8	98·0
Meat	20 ,,	15·0	15·5	103·0
,,	20 ,,	15·0	15·5	103·0

[1897], but in recent years it has not received much attention. It is, however, an admirable reagent for the purpose in hand: (1) because it is easily prepared and kept sodium-free; (2) because it gives a beautiful protein precipitation; (3) because the proteins are not precipitated as the sodium salts. 5 cc. of milk are placed in a 50 cc. flask and diluted to about 15 or 20 cc. with distilled water. 10 cc. saturated bromine water (for human milk) or 15 cc. (for cow's milk) are then added, and after shaking the volume is made up to 50 cc. and the suspension filtered through an ash-free paper. Slight excess of bromine water does no harm and the filtrate should be yellow. 1–1·5 cc. of the filtrate are taken for each estimation, the phosphates removed with zinc acetate in 50 % alcohol and the sodium determined by precipitating it with uranyl zinc acetate in the manner already described. In Table I are shown the results obtained when this method was compared with an incineration method and in Table II the recoveries of known amounts of sodium added to milk.

(5) *Solid tissues.* Incineration is the standard method and has been practised in various forms by almost everyone who has studied this subject. It presents no difficulties provided a suitable temperature can be maintained. The uranyl zinc acetate method of determining sodium was shown in the previous paper [McCance and Shipp, 1931] to be satisfactory for hydrochloric acid extracts of incinerated material. There is therefore no need to discuss this point further here.

5–10 g. of finely minced tissue (1–2 g. of dried material) are placed in a

silica crucible, diam. 6·5 cm., height 4·5 cm. If not dry the tissue is then dried off on a sand bath. This process is accelerated by adding alcohol.

Although not essential an electric muffle furnace with graduated resistance is undoubtedly the most convenient method of effecting the incineration. We have, however, used the following apparatus quite successfully. A layer of iron filings 5 mm. deep is placed in an iron crucible 6·0 cm. high by 9·0 cm. wide. On this layer stands a nickel crucible 7·5 × 7·5 cm. fitted with a porcelain lid. Round the top of this crucible 4 holes are drilled 1 cm. in diameter. This allows access of air to the interior. The annular space between the iron and nickel crucibles is packed with iron filings 3 cm. deep and the filings are covered with a layer of asbestos fibre. The silica crucible which contains the material to be ashed stands inside the nickel crucible. The iron crucible is heated by a 1″ Fletcher burner. The whole is inexpensive and the only part requiring occasional renewal is the external iron crucible.

At the commencement heat is gently applied till all fuming has ceased. The nickel crucible is then covered and heating continued strongly for half an hour. The whole is then allowed to cool, the silica crucible is removed and placed on a large sheet of white paper and the charred mass broken up with a glass rod, any scattered fragments being replaced. The crucible is then replaced and heating continued with occasional stirring of the powder till the residue is white or almost so, otherwise the sodium is not extracted from the ash. The residue in the crucible is then repeatedly extracted with $N/2$ HCl and made up to 100 cc. after cooling: 1 or 2 cc. are taken for the sodium estimation.

Sodium added to tissues may be recovered quantitatively by this method (Table II). Each investigator must satisfy himself that the incineration temperature he employs will not cause volatilisation of the sodium.

Summary.

The technique of a micro-determination of sodium is described (*a*) without incineration in blood-serum, milk, cerebrospinal fluid and urine, (*b*) in solid tissues by incineration.

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